MEMOIRS OF THE SOCIETY FOR ENDOCRINOLOGY

No 4

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THE COMPARATIVE ENDOCRINOLOGY OF VERTEBRATES

PART I

THE COMPARATIVE PHYSIOLOGY OF REPRODUCTION AND THE EFFECTS OF SEX HORMONES IN VERTEBRATES

Proceedings of a conference
held at the Department of Zoology
University of Liverpool
from 12 to 16 July 1954

Edited on behalf of the Society for Endocrinology by
I CHESTER JONES & P ECKSTEIN

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FOREWORD

The Symposium on the Comparative Endocrinology of Vertebrates the proceedings of which are recorded in *Memoirs of the Society for Endocrinology* Nos 4 and 5 would not have been possible without the help of many people and I should like to acknowledge here my gratitude and indebtedness to them The idea of holding the Symposium occurred to me early in 1953 and it was warmly welcomed by Prof Pumphrey and Prof Zuckerman both of whom continued to support me despite the many trials and tribulations which the venture met from time to time A Commonwealth Fund Travel Grant enabled me to visit the United States in the summer of that year and there I was greatly encouraged by the enthusiasm of Prof Hisaw and Prof Greep of Harvard Prof Leathem of Rutgers and Prof Meyer of Wisconsin for an international meeting of endocrinologists prepared to devote a major portion of a conference to vertebrate classes below the mammals Such a gathering became a real possibility when the interest of Dr L Levin of the National Science Foundation and of Dr G Pomerat of the Rockefeller Foundation was aroused and generous grants from these foundations allowed delegates to come to Liverpool from Canada U S A and Europe and when in this country the Royal Society gave grants to help not only travel but also publication expenses Finally the success of the actual meeting itself was ensured by the efficiency of the Chairmen Prof Zuckerman and Dr Folley of my co-editor Dr Eckstein and by the unflagging zeal of my research students Miss Christianson Miss Fowler Mrs Lawson Mr Wright and Mr Phillips and of the laboratory staff Mr Grainge and Mr Alden

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CONTENTS

PROFESSOR R J PUMPHREY F R S

Opening remarks

Ladies and Gentlemen

Although I am only a neurophysiologist myself and have no standing on the course which you are now about to run I am particularly pleased that this Symposium is taking place in a zoological laboratory and I should like to tell you why

In any field in which our ignorance is considerable a pithy remark which seems to embody some guiding principle is certain of a wide currency It was almost exactly twenty years ago that C N Long then in Philadelphia coined the epigram The pituitary is the conductor of the glandular orchestra This observation is still quoted and still seems apt but it has I think acquired a *new look* Then—in 1934—dictatorship was in the air even in the United States where another Long the late Senator Huey was at the peak of his extraordinary career Today we think more democratically Today if I have the picture correctly the pituitary conducts by permission and each member of the orchestra has a clause in his contract entitling him to switch the conductor off when he has had enough The concept of feedback which has overrun neurology is obviously casting its long shadow over endocrinology But perhaps the most surprising thing about this generalization of Long s was that the questions it suggested were at the time not even asked *The* pituitary—but which? The human the canine the mammalian? Was it perhaps true of all vertebrates? What of *Ammocoetes* or Ascidia? And how did it come about? For conductors require a long apprenticeship as well as a reasonably docile orchestra These questions could have received no satisfactory answer then Perhaps they cannot now But it seems surprising—looking back—that they were not asked and the fact that they are being asked today is a measure of the change of emphasis which has taken place in twenty years The truth is I imagine that Long was not interested in evolution or even primarily in endocrinology He was interested in diabetes and most of those who heard or quoted him were interested if not in diabetes in geriatrics or gynaecology or whatever other branch of medicine the endocrines impinged on

Now that much more recently P B Medawar has produced another aphorism—as far-reaching and perhaps as equivocal—to the effect that *it is not hormones which have evolved but the uses to which they are put* it is tempting to speculate how that too will look in twenty years time It is obviously not a crude question of its truth or falsity but an inquiry into the sense in which it is true now and whether it will still be held to be both true and relevant in the same sense in the future

At any rate it seems obvious that the investigation though quite fundamental to endocrinology is a comparative matter proper to zoological laboratories and that the persons best fitted to investigate it are zoologists with an aptitude for endocrinology

rather than say clinicians whose interest in animals other than man is tepid To those of you from overseas where comparative endocrinology has long established itself as a branch of zoology in its own right I may seem to be emphasizing the obvious but that has not been the case in England where comparative endocrinology dissociated from medical and veterinary applications exists on sufferance When this Symposium was first mooted there were plenty of gloomy prognostications It was said—believe it or not!—that there was not enough comparative endocrinology to make a programme It was said that even if such a programme could be constructed no one would be sufficiently interested to come and sit through it Your presence here—for most of you are eminent and many of you have come great distances—is a sufficient answer to the false prophets but they seemed forbidding at the time And I should like to pay my own tribute to Dr Chester Jones for his courage in disregarding them and to today s Chairman for his unostentatious but consistent and far-sighted support of the project from the beginning

Your Symposium therefore promises to be the dawn of a new day for comparative endocrinology in England I believe I shall be speaking for every zoologist in the country in wishing it and you success

PROFESSOR S ZUCKERMAN C B F R S

Chairman s opening remarks

The scope of this Symposium is given by its title and by those of the papers that we are to hear I cannot recall any endocrinological symposium of recent years which has attempted to range over so wide a field We not only have the opportunity of trying to construct an up-to-date picture of the comparative endocrinology of vertebrates but also to put old biological problems into the perspective of modern endocrinological concepts and at the same time to relate newer ideas in endocrinology to the facts of zoology We are breaking from fashion in focusing on the comparative aspects of the subject and I am sure that the experience will prove most rewarding

REPRODUCTION IN TELEOST FISH

By W S HOAR

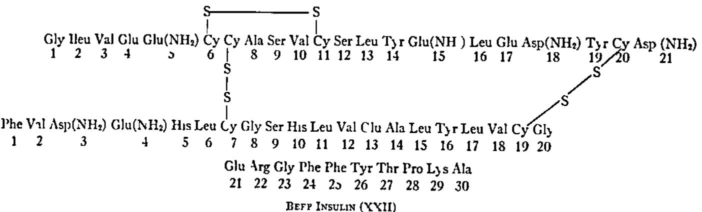
Teleost fish have exploited in one way or another the whole gamut of reproductive devices described among vertebrate animals The members of this highly successful group are usually dioecious but monoecious individuals have been described and some species of the Sparidae and Serranidae are invariably hermaphroditic and self-fertilizing [van Oordt 1933 D Ancona 1949] Again oviparous reproduction is the rule but viviparity has reached a varied degree of specialization Superfoetation is common and in one viviparous species (*Heterandria formosa*) as many as six or more broods regularly develop simultaneously in the ovary [Turner 1937] In another (*Cymatogaster aggregatus*) females give birth to sexually mature individuals [Turner 1938] Males and females are sometimes indistinguishable but all stages of sexual dimorphism are found and this condition may be so extreme that the male (*Photocorynus spiniceps*) is parasitically attached to the female [Norman 1947] Sexual products are often broadcast in the water but elaborate behaviour of migration nest-building brooding and prolonged parental care are likewise familiar phenomena

The chemical co-ordination of this complexity of events is dependent upon a complement of characteristic vertebrate hormones In teleosts as in other vertebrates the gonads and the pituitary gland are primarily involved Bilateral gonadectomy not only renders the animal sterile but prevents the development of secondary sex characters and behaviour associated with reproduction [Zahl & Davis 1932 Regnier 1938 Aronson 1951 Jones & King 1952] Partial castration may interfere with sexual development but does not prevent it [Craig-Bennett 1931 Kobayashi 1951] The implication is that these organs produce hormones responsible for the specialized structures and behaviour associated with reproduction Since the effects of castration may be partially counteracted or prevented by the use of oestrogens or androgens [Grobstein 1948 Aronson 1948] and since these gonadal steroids affect the sexual development of immature fish it is indicated that the hormones involved are chemically similar to those of the higher vertebrates

Sexual development is also dependent upon the pituitary gland Gonads and secondary sex characters fail to develop in hypophysectomized juvenile fish or show a regression if the operation is performed on adults [Vivien 1941 Pickford 1953] Pituitary gonadotrophic activity is likewise evident in the prompt response of fish to injections of minced pituitaries or extracts of the glands In this way premature sexual development and spawning have been achieved in both male and female fish Numerous citations given in an earlier review [Hoar 1951] and in Pickford s [1953] careful investigation of the pituitary of *Fundulus* demonstrate that the gonads and the pituitary

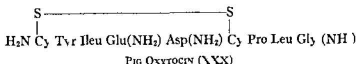
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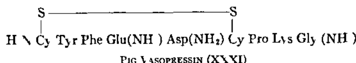


BEEF INSULIN (XXII)

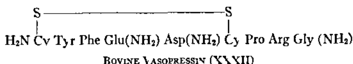
type of muscle preferred may perhaps be determined by the 2 amino acids by which they differ



Pig Oxytocin (XXX)



Pig Vasopressin (XXXI)



Bovine Vasopressin (XXXII)

Du Vigneaud (13) succeeded in isolating another type of vasopressin (XXXII) from the pituitary gland of cattle This has the same composition as that of hogs except that arginine is substituted for lysine In all probability this difference is not important for the action of the hormone It seems to originate from a difference in genes While cattle apply arginine as basic amino acid hogs use lysine and as they have done in the past they will do so in the future

The hemoglobins behave similarly They differ in various animal species as immune reactions and crystal forms prove Nevertheless they all have the same function that is to transport oxygen

The hemoglobin of the horse has been extensively investigated Its molecular weight is assumed to be 66 000 and it contains 543 residues of 18 different amino acids This means that one can calculate at least $6\,4 \times 10^{1}$ different hemoglobins all having the same composition as that of the horse hemoglobin but differing in the arrangement of the amino acids Actually only one of these possibilities is realized in the horse hemoglobin However other types of hemo globin have not been analyzed sufficiently They have about the same molecular weight and the amino acid composition appears to be similar It is certain that they must be arranged in a different manner as one may conclude from the N terminal amino acids

The hemoglobins of horses and mules consist of 6 chains that of humans of 4 or 5 that of cattle and sheep of 4 In the hemoglobins of horses mules and humans all chains end with valine in cattle and sheep 50 per cent of the hemoglobin chains end with valine the other half with methionine This seems

to have been the case in all generations of horses cattle, mules etc , and must therefore be expected to continue unaltered in the future (14)

It is remarkable that human hemoglobin shows a variation that causes sickle cell anemia it occurs in Negroes and is ascribed to a definite gene The oxygen free form of this hemoglobin is much less soluble than the normal one It also differs in other respects Its isoelectric point is shifted more to the alkaline side Apparently this hemoglobin contains more basic and less acidic amino acids than the normal one although this has not yet been proved by analysis As of now only 4 amino acids have been found to differ (15)

One has the impression that in spite of the huge number of possible combinations of amino acids only a few are realized and these are being maintained by nature Of course it could be possible that in the past millions of years other combinations may have existed but did not prove to be suitable Or perhaps there is some ordering principle still unknown to us, which chooses from among the possible combinations

The enzymes probably show similar relations the enzymes of the respiratory chain show about the same effect everywhere However the amino acids may be distributed differently and here and there they may replace each other The above mentioned investigations of Tuppy in Vienna on the sequence of the amino acids in cytochrome c give facts which support this theory (11) Nevertheless there must always be a core in the structure which guarantees the action of the enzyme Pepsin too wherever it occurs, shows about the same action Here the amino acid contents can also vary according to the animal in which the enzyme occurs The investigations have not progressed far enough but in some instances the differences have been detected by immune reactions

A second important group of macromolecules are the nucleic acids especially desoxyribonucleic acid Recently it has stimulated lively discussions since Avery and his co workers (16) succeeded in converting one type of pneumococci into another by adding the nucleic acid of this other type to the culture This transforming factor is nothing else than desoxyribonucleic acid The nucleic acids appear to have a simple structure consisting of only 6 or 7 building stones 4 or 5 purine and pyrimidine bases a pentose and phosphoric acid First a base a pentose and phosphoric acid unite to form a nucleotide, and then many of these together form nucleic acid The type of nucleotide formed depends on the base used Until now it has been found that most nucleic acids contain 4 different bases, several have 5 The nucleic acid of herring sperm is one that contains 5 Its molecular weight is about 1 000 000 one molecule, therefore comprises a total of about 3 000 nucleotides

Desoxyribonucleic acid occurs only in the cell nucleus and has approximately the same composition in all animals The differences among the various species

can only be the arrangement of the nucleotides that is to say the sequence in the nucleic acid chain Here too is a great number of possibilities Surely not all of them are realized The existing ones will be handed down from generation to generation of the various animals plants bacteria and viruses

In this connection the third group of macromolecules—the blood group substances—are of special interest because they reveal differences between human races and differences in the liability to illness Some diseases strike the individuals of a certain blood group more frequently than those of the others From the chemical point of view these substances are interesting because they consist of sugars amino sugars and about 10 kinds of amino acids Many different molecules could be built up depending on what kind of building stones are used But as far as we can judge from present knowledge the blood group substances differ from one another quantitatively more than qualitatively and their quantitative differences are maintained within the members of a blood group According to Rondle and Morgan the relation of glucosamine to galactosamine is 1 6 in A_1 2 2 in A and 2 8 in B (17)

Therefore it seems that hereditary characteristics can be represented by different quantitative ratios and perhaps this applies not only to the constituents within a molecule but also to the molecules within a cell Florkin (18) has found that the pattern of free amino acids in blood and tissue differs from one species of animals to another A further example is found in the work of Lehninger (19) on the synthesis of ascorbic acid All animals which do not depend on their food in this respect transform D glucose easily into L ascorbic acid But to our surprise Lehninger has demonstrated that the enzymes involved occur also in the liver of monkey and probably also of guinea pig and man for which ascorbic acid is a vitamin All these animals can perform the first steps of synthesis namely the production of L gulonic acid from glucose and the oxidation of the latter to the hypothetic 3 keto L gulonic acid The last step does not occur in the monkey liver the involved enzyme is present but it has no opportunity to act because of another enzyme of much higher activity which decarboxylates 3 keto L gulonic acid to L xylulose The two enzymes compete for the same intermediate and only the preponderance of the decarboxylating one prevents the monkey from producing ascorbic acid This unfavorable quantitative ratio is genetically controlled and is thus preserved through the generations

Now let me consider a further possibility of variations Most of you are familiar with the work of Pauling (20) on the macromolecular structure of collagen and with that of Watson and Crick (21) on desoxyribonucleic acid The peptide chains and the nucleotide chains are assumed to be coiled in helix like spirals The chains of non fibrous proteins are bent or folded in a different manner the nature of which we do not yet know precisely However we can easily imagine that different macromolecules must arise depending upon the type

of folding and the processing of the spirals The biological activity of a macromolecule depends on those side chains of its links which are situated at its surface

This manner of variation could be called a "variation in space' which applies also to the structure of the cell particles the nuclei, the mitochondria and the microsomes Their structure and composition vary among the different species and among the organs within one species Roka and co workers (22) showed that the enzyme pattern of the mitochondria varies widely among the different organs

The nucleus contains information about species and breed that the future organism will have In Frankfurt we are especially interested in the nucleus of fish sperm It has a volume of about 7 μ^3 and is very compact so that no structure can be detected by either the light or the electron microscope There is barely room for water and according to our investigations, it consists of nucleoprotamine and nothing else

From this nucleoprotamine not only the chromosomes but also the genes must be formed and each gene must have at least one nucleoprotamine of its own There are from 4 5 to 5 million molecules in a single nucleus and, since both parts of the nucleoprotamine—the nucleic acid and the protamine—are not homogeneous substances but consist of several components several millions of nucleoprotamines could be formed according to which components are combined together One molecule of nucleic acid binds about 100 protamine components These could be arranged in different sequences along the nucleic acid chain This provides a further possibility for variation More details will be found in chapter 14

Perhaps another point may be added that is the distribution of the nucleoprotamine molecules within the nucleus They are not scattered at random but are arranged in a very definite structure The nucleoprotamines form fibers and these fibers are arranged in bundles or lamellae as shown in microphotographs of Yasuzumi (23) Rebhun (24) and Ris (25)

One additional fact must be mentioned In these nuclei no metabolic reactions take place because they do not contain enzymes and do not consume oxygen They are merely structure and in it must be contained all potential qualities which the male fish transmits to his offspring Otherwise there is always action where there is life According to geneticists the nuclei of the egg cell have the same composition as those of the spermatozoa they must therefore also consist of structure Whenever the two structures unite action revives

Such considerations on the order in space apply also to the order in time for the metabolic reactions I mentioned at the beginning that the fundamental reactions are the same in almost all organisms but presumably they follow each other in a different order Thus life is architecture and melody at the same time

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CHAPTER 2

Current Ideas of the Structure of Deoxyribosenucleic Acid

*Robert L. Sinsheimer**

This chapter will be concerned with the current status of our knowledge of the structure of deoxyribosenucleic acid—DNA. Specifically it will be concerned first with the available knowledge of the basic structure of DNA together with the implications of this structure for certain theories of DNA replication, then with the available knowledge of nucleotide sequence in the DNA structure and finally, with the problem of the heterogeneity of DNA.

In a sense this is an awkward time to consider problems of DNA structure. The status of our knowledge with regard to DNA is very similar to that which comprised our knowledge of proteins for some three or four decades. By 1910 it had been conclusively established that proteins—at least the simpler proteins—are essentially composed of polypeptide chains. But this information while clearly of fundamental importance did not really help very much to understand how proteins accomplish the remarkable feats of catalysis and contraction and the other functions that they perform. And indeed it required some four decades and the successive development of techniques for the isolation of pure proteins, the development of techniques of specific enzymatic degradation and chromatographic separation of protein fragments, and the continued refinement of X-ray diffraction analysis before the present stage of protein chemistry was attained, wherein at last the amino acid sequences in a few proteins are being deciphered, people have hopes of learning the spatial orientation of these amino acids, and one can begin to think definitively about the linkage of structure and function.

Our knowledge of DNA is almost back in the 1910 stage of protein chemistry with the additional handicap that one is not really sure just what function one should ask of these molecules. While the accumulated experience of protein

* The major part of this chapter was prepared while the author was a member of the Department of Physics at Iowa State College.

chemistry will be of great help one may suspect that it will still require many years before that which should be most interesting about DNA structure—its relation to function—can be intelligently discussed

However, much has been learned in the last ten or even five years A basic structure has been proposed for DNA which is almost certainly correct This is with slight but important modifications, the two strand helix originally described by Watson and Crick (1) (Fig 1)



Fig 1 —Molecular model of the two strand helix of DNA (Courtesy of Dr L D Hamilton)

In its current form (2) this structure consists of two polynucleotide chains intertwined about each other—the period of the twist is 34 A and there are ten nucleotides in each chain per twist The backbone of each chain is composed of deoxyribose residues linked by phosphate groups between the 5 carbon of one residue and the 3 carbon of the next The two chains run in opposite directions—both are right hand helixes

The purine and pyrimidine bases are directed inward from the deoxyribose residues The variety of purines and pyrimidines usually found in DNA preparations is shown in Figures 2 and 3 In the two strand helix the bases of one chain are linked through specific hydrogen bonds to those of the opposite chain The structure postulates a complementarity between the bases of the two

chains adenine is always opposite thymine (Fig. 4) and guanine is always opposite cytosine (Fig. 5). Thus specification of one chain would completely specify the other so that the information presumed to be represented by the sequence of nucleotides is present twice. The question of complementarity will be considered in more detail below.

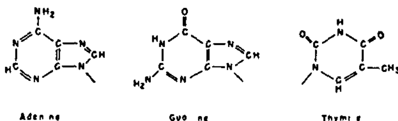


FIG. 2.—Three heterocyclic groups found in all DNA preparations.

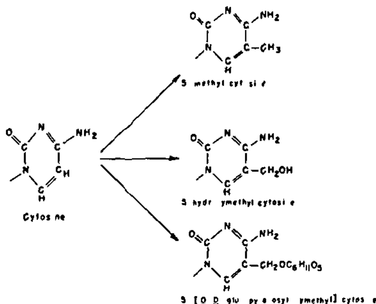


FIG. 3.—Cytosine and the various substituted cytosine rings found in some DNA preparations.

It is worthwhile to inquire into the evidence for this interesting structure. The structure was originally developed to provide a qualitative agreement with the X-ray diffraction patterns of DNA fibers obtained by Wilkins *et al.* (3, 4) and by Franklin and Gosling (5). This X-ray work had shown that DNA fibers could assume two degrees of regularity. The less regular—observed best at high humidity—gives rise to the B pattern (Fig. 6) with its cross characteristic of a helix. The more regular form, obtained only under certain conditions

of humidity gives rise to the A pattern a nematic crystalline order from which it has been possible to make Patterson projections

That the proposed structure—after considerable revision of the exact atomic positions and spacings (2 6)—accounts for the observed patterns is now well established However, the pattern arises from only the ordered regions of the fibers and hence can logically be said to provide information about only

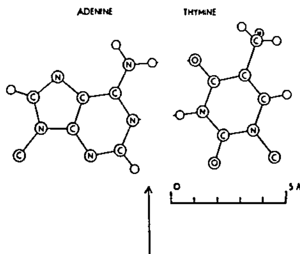


FIG 4 —Complementary pairing of adenine and thymine in DNA

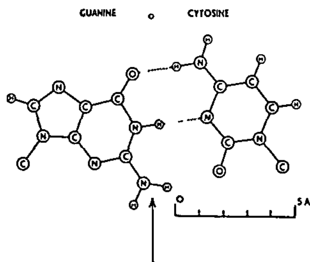


FIG 5 —Complementary pairing of guanine and cytosine in DNA

the ordered regions Estimates of the fraction of the fibers which is ordered range from 5 to 50 per cent

More conclusive evidence for the generality of a two fibered structure is provided by the recent degradation experiments of Thomas (7) and of Schumaker Richards, and Schachman (8) Both groups investigated, by physical means the kinetics of the enzymatic degradation of DNA with pancreatic deoxyribonuclease On the assumption that the enzyme breaks only one internucleotide link at a time, the rate at which the particle size as measured by



FIG 6 — B X ray diffraction pattern from DNA fibers (4 5)

light scattering or by viscosity will decrease will be very different depending upon whether the structure concerned is a single or a double strand If it is a single strand every cut will break the chain If it is a double strand two cuts on opposite chains must be made sufficiently close together that the hydrogen bonds of the nucleotide pairs between cuts are no longer sufficient to hold the chains together before the particle will be severed Indeed, it is possible from the data to estimate the minimum number of nucleotide pairs which will be adequate to hold the chains together These effects of structure will evidently be most marked in the initial stages of degradation

The manner in which the molecular weight as measured by light scattering, falls off as a function of the number of links cut by deoxyribonuclease is shown in Figure 7 The experimental curve is clearly different from that expected for a single chain and instead, matches rather well that expected for a double chain when two hydrogen bonded nucleotide pairs are adequate to hold the chains together—i e two cuts must either be directly opposite or within one nucleotide pair to sever the double chain

Figure 8 from Schumaker, Richards and Schachman (8), is a plot of 1 minus the ratio of the relative viscosity of the degraded DNA solution to the initial relative viscosity of the solution as a function of time when the numbers of bonds cut has been shown to be a linear function of time From theory

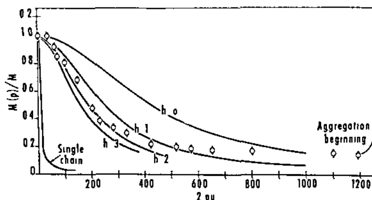


FIG 7 —Fractional decrease in molecular weight of DNA as a function of the number of bonds broken by deoxyribonuclease The points were determined by the light scattering-titration method the lines are calculated from double-chain degradation theory *h* is the number of hydrogen bonded nucleotide pairs assumed to be necessary to hold the two chains together (7)

for a simple double chain structure the slope of the line joining the data points would be 2 00 Actually it is 1 47 It is possible to account for this result in various ways An admixture of about 5 per cent of single strands could cause this result—or conversely one can say that this DNA preparation must contain less than 5 per cent of single stranded structures Or the presence of a small number of preformed single breaks in the double chain—preformed in *the sense of being present prior to this experiment* without prejudice as to whether they were intrinsically present in the DNA or were introduced during isolation—could account for this result Calculations indicate that an average of one such break per 3 000 nucleotide links would be adequate to reduce the slope from 2 to the observed 1 47—or conversely there can be no more than one preformed break per 3 000 nucleotides if the double strand hypothesis is accepted

Many other interpretations of these data are possible if the presence of triple stranded, or more complex, structures is admitted but there is no evidence for such structures, and they are ruled out for the ordered regions by the X ray data

The data of Schumaker, Richards, and Schachman also lead to the conclusion that only a small number—between 0 5 and 5 0—of hydrogen bonded *nucleotide pairs is necessary to hold the chains together*

Additional data on the action of heat (9) and of acid (10) on DNA are also most effectively explained by the assumption of a general two stranded structure The usually found molar equality of adenine and thymine and of guanine

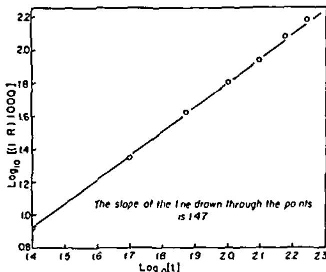


FIG 8 —Decrease in viscosity of a DNA solution as a function of the time of action of deoxyribonuclease *R* is the ratio of the relative viscosity of the DNA solution at time *t* to its initial relative viscosity (8)

and cytosine (or cytosine plus substituted cytosines) (11) is also of course neatly explained by this complementary two stranded structure

The evidence seems conclusive that this two stranded structure is valid for the DNA preparations that have been examined It is, however, neither necessary nor proper to assume that DNA is in this form at all times The DNA preparations that have been studied by physical means have been either from mature cells such as thymus or from cells with a migratory mission such as sperm or phage It is at least conceivable that at various stages of the mitotic cycle DNA may assume other forms There is just enough evidence such as the repeated occurrence of DNA fractions with non complementary base ratios obtained from fractionation experiments done in various ways (12 13), to suggest that there may be some important exceptions hidden behind an over all complementarity

The postulate of complementarity must be examined in more detail. Strictly complementarity would require that DNA be composed of only four nucleotides—deoxyadenylic, deoxyguanylic, deoxycytidylic and thymidylic acids. Actually almost all DNA preparations examined are composed of five kinds of nucleotides, the most common variation involves a substitution upon the 5 position of cytosine as in 5-methylcytosine (14), 5-hydroxymethylcytosine (15), and the glucose substituted 5-hydroxymethylcytosine (16, 17, 18). There is also found in small amounts in many DNA's of bacterial origin a variant of adenine, 6-methylaminopurine (19). In general it is found that the molar content of cytosine plus the substituted cytosine equals the molar content of guanine in the DNA, and that of adenine plus 6-methylaminopurine equals

TABLE 1

COMPARISON OF DISTRIBUTION OF DEOXYCYTIDYLIC ACID (C) AND 5-METHYLDEOXYCYTIDYLIC ACID (M) AMONG DINUCLEOTIDES OBTAINED FROM CALF THYMUS DNA BY DIGESTION WITH PANCREATIC DEOXYRIBONUCLEASE

C Dinucleotide	Mole Fraction of Digest	M Dinucleotide	Mole Fraction of Digest
Cp-Cp	1.11	Mp-Mp	0
CpTp-	0.78	Mp-Tp	0
Tp-Cp	2.34	Tp-Mp-	0
Cp-Ap	0	Mp-Ap	0
Ap-Cp	3.22	Ap-Mp	0
CpCp-	0.[illegible]5	Mp-Gp	0
Gp-Cp	0.12	Gp-Mp-	1.03

that of thymine (with the exception of the pathological stage which occurs when thymine requiring cells are grown in the absence of thymine).

The presumed complementarity of DNA has been used as a basis for proposed schemes of DNA replication. It has been suggested that the two chains come apart (20)—perhaps only in short segments at a time—and that each serves as a template upon which to form its complement, this process resulting in two double chains each identical with the first. Various methods (21–25) for accomplishing this feat have been proposed, resulting in somewhat different distributions of the parental substance among the progeny molecules.

However, the presence of a fifth nucleotide disturbs this argument unless it is presumed that the modified nucleotide appears at random. There appears to be clear evidence that this is not the case. Studies of the comparative distribution of cytosine and 5-methylcytosine among the dinucleotides (26) obtained from calf thymus DNA by the action of pancreatic deoxyribonuclease reveal striking differences (Table 1). Again, the distribution of 5-methylcytosine

among fractions of calf thymus DNA obtained by the method of Chargaff *et al* (27) is distinctly non uniform (Table 2)—one fraction had no 5 methylcytosine Considering the number of nucleotides involved such a fluctuation would be most unlikely to be a purely statistical result

An even more convincing argument against a simple complementary replication of DNA appears from the following data In the DNA of T2 bacteriophage there is no cytosine it is replaced by 5 hydroxymethylcytosine (HMC), and 77 per cent of this HMC is substituted with a glucose on the hydroxymethyl group (28) Levinthal has shown that the DNA of T2 is composed of one large piece (29), comprising about 40 per cent of the DNA and a number of smaller pieces Brown and Martin (30) have fractionated the DNA of T2

TABLE 2

MOLAR PROPORTIONS OF PYRIMIDINES IN CERTAIN DNA FRACTIONS

	Fraction			
	1	2	3	4
Moles per 100 gm atoms of P				
Pyrimidines recovered	41 2	52 2	45 /	46 3
Corrected mean proportions				
Thymine	24 2	24 0	29 0	28 7
Cytosine	23 9	23 4	19 8	21 3
5 Methylcytosine	1 9	2 /	1 2	0†
Molar ratio				
Thymine to 5 methylcytosine	12 7	8 9	24 2	
Cytosine to 5 methylcytosine	12 6	8 7	16 5	

Th[illegible] m[illegible]les [illegible]f pyr[illegible]m[illegible]d[illegible]es act[illegible]lly f[illegible]d per 100 gm [illegible]t[illegible]m[illegible] [illegible]f phosphor[illegible] a[illegible]e [illegible]epo[illegible]ted [illegible] the first l[illegible] Th[illegible] m[illegible] [illegible]p[illegible]port[illegible] [illegible]f [illegible]b[illegible] [illegible]t[illegible]t[illegible]e[illegible] [illegible]t ha[illegible] be[illegible] [illegible]ect[illegible]d on th[illegible] ass[illegible]mpt[illegible] that h[illegible]lf th[illegible] n[illegible] [illegible]ic[illegible] [illegible]d phosph[illegible]rus [illegible]s [illegible] [illegible]trib[illegible]ted by th[illegible] pyr[illegible]m[illegible]d[illegible] [illegible]leot[illegible]des

† [illegible] hyd[illegible]ly[illegible] [illegible] [illegible]perime[illegible]t w[illegible]th F[illegible]ct[illegible] 4 [illegible] [illegible]m[illegible] [illegible]t [illegible]am[illegible] [illegible]t [illegible]f [illegible]m[illegible]thylcytos[illegible] ([illegible]bo[illegible]t 0 6 m[illegible] [illegible]) was fou[illegible]d ([illegible]7)

on a histone column into two components and they have shown (31) that their fraction A is Levinthal s large piece while their fraction B is composed of the small pieces Further they have shown that the non glucose substituted HMC is confined to the large piece Only about 65 per cent of the HMC of the large piece is so substituted while 100 per cent of the HMC of the small pieces is glucose substituted Since all the DNA components are replicating in the same cell at the same time, it would seem unreasonable to ascribe the limited glucose substitution to a metabolic deficiency On the contrary it would seem that one must conclude that the replicating mechanism, whatever it may be, knows just when to insert an ordinary HMC and when to insert a glucose substituted HMC to duplicate the original phage particle—for the glucose content of T2 remains constant over many generations

Recently the matter of the heterogeneity of DNA has been the subject of

various investigations If one prepares DNA from a calf thymus there is no reason at all to believe that this is a homogeneous substance The DNA content of one calf thymus nucleus is equivalent to several hundred thousand molecules of molecular weight 8 000 000 to use a fashionable number for the particle weight of DNA preparations Very likely a calf thymus DNA comprises many thousands of molecular species

This being the case, any bulk data obtained upon such a preparation can be of only statistical significance Thus limited data have been obtained about the relative molar proportions of various nucleotide sequences in calf thymus DNA In this way the nucleotide sequence about some 10 per cent of the phosphate links can be specified at present (*Table 3*) (*32 26*) and it is likely that this could be raised to perhaps 30 per cent with an extension of existing techniques But these data can have only statistical significance

TABLE 3

MOLAR PROPORTIONS OF DINUCLEOTIDES IN THYMUS DNA DIGEST

Y	X				
	M	C	T	A	G
M	0		0	0	0
C		1 11	0 78	0	0 7›
T	0	2 34	1 38	0 10	0 16
A	0	3 22	1 36	0 46	0 9ʹ
C	1 03	0 12	? 61	0 20	0 8?

M 5-methyldeoxycytidylic acid C deoxycytidylic acid T thymidylic acid A deoxyadenylic acid G deoxyguanylic acid XY = X—p—Y—p where Y refers to the nucleoside carrying the phosphate monoester group in each case

DNA preparations from varied organisms (11) particularly various species of bacteria vary in the ratio of adenine to guanine (or thymine to cytosine) from 0 4 to 2 7 (33) but again this is only an average over many species of molecules

That large molecular weight DNA fractions of varied nucleotide composition can be obtained from preparations such as calf thymus DNA was first demonstrated by Chargaff *et al* (27) By successive extraction of a chloroform nucleohistone gel with increasing concentrations of salt high molecular weight fractions were obtained with increasing adenine to guanine ratio (34, 35) Lucy and Butler (12) demonstrated that even successive extractions of such a gel with the same salt concentration would yield fractions of varied composition More recently methods of column fractionation have been applied to DNA Brown and Watson (36) using histone columns have obtained varied fractions (Fig 9) from several DNA preparations As mentioned above, Brown and Martin (30) have fractionated T2 DNA into what appear to be the large and

small pieces first demonstrated by Levinthal's autoradiographic technique. Main and Cole have investigated the utility of columns of calcium phosphate gel for DNA fractionation (37).

The most extensive fractionations have been carried out by Bendich and co-workers (13, 38) using modified cellulose columns (Fig. 10). Their successful fractionation of the transforming principle has shown that it is possible quanti-

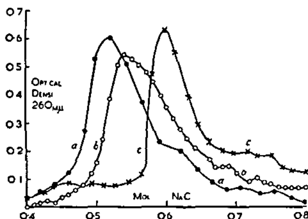


FIG. 9.—Elution diagrams of some deoxyribonucleic acids from histone columns obtained by elution with continuously increasing salt concentration (36). Curve *a*, DNA from *E. coli*. Curve *b*, DNA from calf thymus. Curve *c*, DNA from human white blood cells.

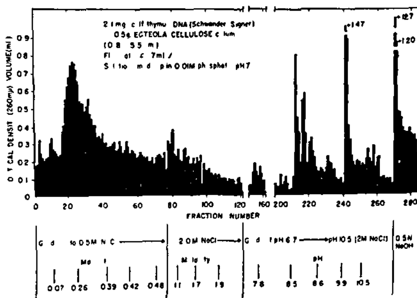


FIG. 10.—Fractionation of calf thymus DNA on a substituted cellulose column (38).

tatively to recover biologically active material from these columns (39) (Successful chromatography of the transforming principle on histone columns has been reported by H Ephrussi Taylor [40]) Unfortunately the cellulose columns are not entirely reproducible and the theoretical basis of these empirical fractionations is not well understood In some instances different fractions have different nucleotide compositions in others, size seems to be an important factor

That DNA preparations contain particles of varying size as well as nucleotide composition was first shown by sedimentation experiments at very low concentrations by Shooter and Butler (Fig 11) (41) Schumaker and Schachman (42) have also demonstrated this heterogeneity The thymus DNA preparations appear to contain particles with a range of sedimentation constant from 10 to 40 or more svedbergs

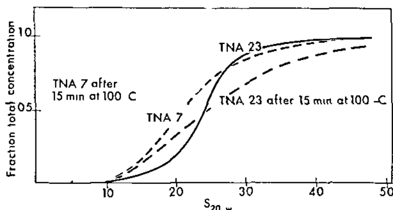


FIG 11 —Effect of heat on the distribution of the sedimentation coefficient of two samples of DNA 0 005 per cent DNA in 0 2 M NaCl (41)

In principle a variation in sedimentation constant could reflect either a variation in molecular weight or a variation in shape and thus in frictional ratio If the latter were the cause of the spread in sedimentation constant of the DNA then the more slowly sedimenting particles should have a greater frictional ratio and hence be a more viscous component However after isolation of some of the slower moving material, it proved to be less viscous than the bulk of the DNA preparation (43) indicating that the spread in *s* is undoubtedly in part the result of a spread in molecular weight over a considerable range

This newer evidence for the heterogeneity of the usual DNA preparations emphasizes the necessity of obtaining homogeneous preparations before the structural study of DNA can proceed very much further—before sequence data can have more than statistical meaning and before really significant physicochemical data can be obtained

To attempt to prepare a homogeneous DNA it would seem logical to try to start with a source that could have only a few—or ideally one—DNA molecule within it The obvious place to look for such a source would be among the bacterial viruses, and among the viruses the smaller ones would be the most appropriate

The smallest bacterial viruses known are S13 and the related φX174 (44) Concerning the latter four lines of evidence have been available to indicate that it is small It produces large plaques (45) which can be interpreted to mean

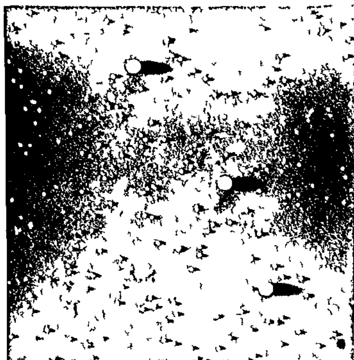


Fig 12—Electron micrograph of aggregates of purified virus φX174 The polystyrene spheres are 132 mμ in diameter

that it has a high diffusion rate Ultra filtration studies through graded membranes suggested a diameter less than 12 mμ (45) X ray (46) and α particle (47) inactivation data have suggested a sensitive volume of about 16 mμ in diameter A crude sedimentation run (48) (the virus suspension was placed in a capillary tube and an attempt made to determine how fast the plaque forming activity sedimented) resulted in an estimated s equivalent to 33 svedbergs this s is not correct but historically it has been important because, if one assumed the virus to have a spherical shape and a reasonable partial specific

volume an s of 33 corresponded to a diameter of 16 mμ in good agreement with that derived from the inactivation data

This virus has now been prepared in our laboratory in pure form in reasonable quantity The virus in monomeric form has a sedimentation constant of $s = 114$ It also has the rather unusual property of forming when purified an aggregate of $s = 155$ An electron micrograph of the virus is shown in Figure 12

From light scattering data the particle weight of the aggregate is about $25\ 7 \times 10^6$ For the monomer a value of $6\ 2 \times 10^6$ has been obtained The virus contains DNA and no RNA The DNA content is about 25 per cent indicating a total DNA content of $1\ 6 \times 10^6$ in molecular weight units Light scattering and sedimentation data obtained upon DNA isolated from the virus suggest that it is present as a single particle

In summary conclusive evidence is now available that the Watson Crick structure is a valid structure for the DNA of our usual preparations To obtain more detailed knowledge of DNA structure more homogeneous preparations are needed To obtain such preparations will require better methods of fractionation and more adroit choice of the DNA source

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CHAPTER 3

Enzymatic Synthesis of Deoxyribonucleic Acid

Arthur Kornberg

AMONG the most intriguing aspects of biology are those concerned with the chemical basis of genetics We know that the chromosomes in the nucleus of the sperm and of the egg contain the information that leads to the production of a complex creature From the union of these nuclei a great variety of cell types results and each cell type in turn produces many millions of faithful replicas of itself The distinctive structures and functions of each cell are determined by its chromosomes Whenever a cell is to divide an exact reproduction of its chromosomes is made for the daughter cell One of the magic ingredients that chromosomes are made of is deoxyribonucleic acid or DNA It is the substance which is at once the blueprint and the mold out of which the many copies of the enzymatic machinery of the cell are forged In this paper we shall consider the experimental approaches taken to answer the question how a cell makes its DNA But first I should like to mention briefly the development of techniques and ideas which made some of these studies possible

The work on DNA biosynthesis in our laboratory has been a team effort and I gratefully acknowledge the collaboration of my colleagues Dr I R Lehman Fellow of the American Cancer Society Dr M J Bessman Fellow of the Public Health Service and Mr E S Simms

Our own interest in the enzymatic synthesis of nucleic acids started with attempts to learn about the enzymatic mechanisms that the cell uses for assembling some of the complex nucleotide coenzymes and the simpler nucleotide units of which they are composed In Figure 1 are the structures of two simple nucleotides One—adenosine 5 phosphate a common constituent of coenzymes and one of the four nucleotides in ribonucleic acid (RNA)—contains ribose as the sugar component The other three nucleotides of RNA contain different nitrogenous bases guanine cytosine and uracil The way in which these nucleotides are synthesized enzymatically is now almost completely understood as a

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result of contributions from several laboratories Buchanan s and Greenberg s notable among them The other nucleotide shown in Figure 1 is thymidine 5 phosphate It is one of the four deoxyribonucleotides of which most DNA s are composed The nucleotides of DNA contain 2 deoxyribose as the sugar component and the same nitrogenous bases mentioned earlier except that 5 methyl uracil—that is thymine—replaces uracil

We know relatively little about the enzymatic synthesis of deoxyribonucleotides However there are indications that the deoxynucleotides may be formed by direct reduction of the corresponding ribonucleotides Thus as shown in the

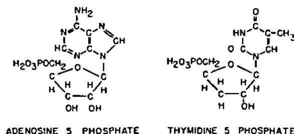


Fig 1

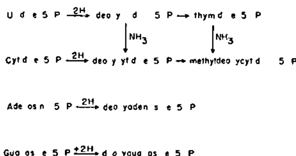


Fig 2 —Hypothetical pathways of deoxynucleotide synthesis

hypothetical schemes in Figure 2 we can conceive of a direct conversion of uridine 5 phosphate to deoxyuridine 5 phosphate of adenosine 5 phosphate to deoxyadenosine 5 phosphate and so on While experimental evidence for these reductive reactions is lacking the current studies of Friedkin (4) provide clear indications that deoxyuridine 5 phosphate can be a precursor of thymidine 5′ phosphate He found and is now purifying an enzyme system from *Escherichia coli* which converts deoxyuridine 5 phosphate to thymidine 5 phosphate requiring hydroxymethyltetrahydrofolic acid as a source of the methyl group and possibly the reductive potential as well

With problems of the enzymatic synthesis of single nucleotides and as we shall point out in a moment of the more complex coenzymes approaching solution the problem of how nucleotides are polymerized into the very long chains —the nucleic acids—can now be studied from this background

First what do we know about the structure of DNA? One of the remarkable facts which emerged from the analytic studies of DNA by Chargaff (3) and others is that the DNA of a given species contains equal amounts of thymine and adenine and equal amounts of guanine and cytosine While the thymine to cytosine or adenine to guanine ratios varied widely from species to species the purine content always exactly equaled the pyrimidine content With the aid of these observations with Pauling s α helix theory (14) and by ingenious deductions from X ray diffraction studies Watson and Crick (17) have inferred a bonding through hydrogen atoms of thymine to adenine and guanine to cytosine (see Fig 3) and have proposed their now widely known model of DNA as a

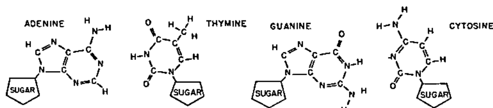


FIG 3 —Hydrogen bonding of bases in DNA

double helix (see Fig 4) In this model the two polynucleotide strands are linked together by the hydrogen bonds uniting thymine with adenine units and guanine with cytosine units In this figure we have the representation of a tiny segment of a DNA molecule containing about 10 nucleotides in each strand According to most estimates a DNA molecule contains about 10 000 nucleotides in each strand and therefore is about a thousand times as long as the fragment pictured here The greatest significance of this model of DNA is that it provides a plausible device for a duplication process According to the Watson and Crick idea the unwinding of the two complementary chains provides a fresh template from which two new chains complementary to each of these can be synthesized

Let us return now to the question of how this synthetic process takes place in the cell Our own studies were based on the hypothesis that the fundamental building block of a nucleic acid is an activated nucleoside 5 phosphoryl unit that is an activated nucleotidyl group such as adenosine triphosphate (ATP) This idea stems largely from the studies of coenzyme synthesis (6) As shown in Figure 5 the formation of diphosphopyridine nucleotide (DPN) involves a condensation of nicotinamide mononucleotide with the adenosine 5 phosphoryl

unit of adenosine triphosphate (ATP) and a consequent elimination of inorganic pyrophosphate Similar condensations lead to the synthesis of flavin adenine dinucleotide and of the immediate precursor of coenzyme A The activation of fatty and amino acids as shown by the work of the Lipmann school (11), follows an analogous pattern and Berg (1) has demonstrated that adenyl acylates are the intermediates in these reactions This reaction mechanism is not re



FIG 4 —Hypothetical helical structure of DNA

stricted to the adenine nucleotide coenzymes but embraces all the nucleotide coenzymes For example as shown in Figure 6 the uridyl coenzymes have through the work of Kalckar and his group (12) been shown to have a similar origin The same mechanism was found by Munch Petersen (13) to hold for the synthesis of guanosine diphosphate mannose a coenzyme unemployed at the

1 ARPPP(ATP) NRP(otm m d m onucleotid) ⇌ ARPPRN(DPN) PP

2 ARPP FRP(bofle n P) ⇌ ARPPRF(FAD) + PP

3 ARPP + p tethe e P ⇌ ARPP po t then + PP
ATP → ARPP pont then (Co A)
P

4 ARPP + RCH_2COOH ⇌ $ARPOCCH_2R$ PP
O

O O

$RCHNH_2COOH$ $ARPOCCHNH_2R$ PP
O

Fig 5 —Synthesis of adenine nucleotide coenzyme

URPPP(UTP) G P(g uc 1 P) ⇌ URPPG(UDPG) PP

2 URPPG G 1 P(g lactos 1-P) ⇌ URPPG 1 PP

3 CRPPP(CTP) Choli P ⇌ CRPP Chol PP

4 GRPPP(GTP) MP(m 1 P) ⇌ GRPPM PP

Fig 6 — Synthesis of uridine cytidine and guanosine nucleotide coenzymes

Ad osine O P(O)(HO) OP(O)(OH) O P(O)(OH) OH

N cot m de ib se O P(O)(O) OH

Fig 7 —Nucleophilic attack of *nicotinamide* mononucleotide on ATP

moment and applies equally well to the cytidyl coenzyme found by Kennedy and Weiss (5) to be active in phospholipid synthesis A helpful and unifying way of regarding these nucleotidyl condensations is as nucleophilic displacement reactions as suggested by Koshland (10) (see Fig 7) An unshared pair of electrons on the oxygen of the attacking group is directed to the nucleotidyl phosphorus displacing inorganic pyrophosphate Applied to the problem of poly

unit of adenosine triphosphate (ATP) and a consequent elimination of inorganic pyrophosphate Similar condensations lead to the synthesis of flavin adenine dinucleotide and of the immediate precursor of coenzyme A The activation of fatty and amino acids as shown by the work of the Lipmann school (11), follows an analogous pattern and Berg (1) has demonstrated that adenyl acylates are the intermediates in these reactions This reaction mechanism is not re



FIG 4 —Hypothetical helical structure of DNA

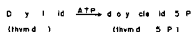
than 0 1 of 1 per cent of the thymidine added and therefore less than 0 001 μmole, it was nevertheless a significant and reasonably reproducible result (9) The labeled product was readily depolymerized by pancreatic deoxyribonuclease and behaved like DNA in a variety of chemical fractionation procedures Of additional interest was the observation that thymidine was converted to phosphorylated acid soluble derivatives which were separated chromatographically and tentatively identified by their migration in the chromatogram as the mono di and triphosphates of thymidine Assuming these nucleotides to be intermediates on the pathway to DNA synthesis we purified the kinases re

TABLE 1

THYMIDINE CONVERSION TO NUCLEOTIDES AND DNA (C P M)

F act	0 M t	60 M tes
Acid insoluble (DNA)	32	196
Acid insoluble–DNAase treated	35	30
Acid soluble		
Thymidine 5 P	<1 000	17 100
TDP	<500	9 500
TTP	<100	2 300

M Thym d C 1 μm l 4 × 10⁶ p m ATP g t g y t m + ATP *F col* t act



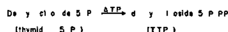




FIG 9 —*Conversion of thymidine to DNA*

sponsible for these conversions and were able to prove that these compounds were indeed thymidine 5′ phosphate thymidine 5′ diphosphate and thymidine 5 triphosphate

We then turned to purifying the enzyme system responsible for the conversion of thymidine to DNA and were able to show that there was a sequence of reactions as delineated in Figure 9 As purification of the system proceeded the reaction went preferentially with thymidine triphosphate and thymidine or thymidine 5 phosphate was no longer reactive The procedure for purifying the enzyme—and this is still the focal point of our efforts—is at present as follows (see Table 2) Rapidly dividing *E coli* cells are harvested and disrupted by

sonic disintegration The extract is then fractionated with streptomycin For further purification a DNAase treatment of the preparation was essential and subsequent to that the use of alumina gel ammonium sulfate and ethanol fractionations have led to preparations which are a thousand times or more enriched with respect to protein as compared with the starting extract The purity of our best preparations is as yet undetermined Our present problem is partly a logistical one and involves the provision of sufficient amounts of this purified fraction as a camp from which to proceed further

During the course of the purification of the enzyme we learned a number of significant things about the reaction itself The disruption of the nucleoproteins by the use of nuclease at stage 3 (Table 2) demonstrated a clear and irreplaceable need for polymerized DNA and at this stage we became aware that the reaction would not proceed to a detectable extent unless all four deoxyribonucleoside triphosphates were present in the incubation mixture (2, 7 8) In the experiments shown in Table 3 carried out under typical assay conditions 0 50 mμmole of DNA nucleotide was produced The isotope in this case was contained in the acid stable phosphate of thymidine triphosphate Omission of the other three deoxynucleoside triphosphates or of even a single one of them results in a complete suppression of the reaction as determined under these conditions Similarly the lack of magnesium ions of DNA or the substitution of a DNA that was first treated with pancreatic DNAase results in no detectable reaction As contrasted with the behavior of cruder enzyme systems, ATP is now no longer essential and its presence (or that of ADP) does not affect the reaction

TABLE 2

PURIFICATION OF ENZYME

Step	Total Units	Units/mg Protein	Overall Recovery (Per Cent)
1 Sonic extract of *E coli*	880	0 12	100
2 Streptomycin fractionation + Spinco centrifugation	1 350	5 7	153
3 Nuclease treatment dialysis lyophilization	1 160	6 8	132
4 Alumina gel fractionation	700	19 0	79
5 Ammonium sulfate fractionation	365	58 1	42

Before proceeding with a detailed consideration of the *E coli* system, it is of interest to mention that Dr Carl G Harford in our laboratory has found what appears to be a similar system in tumor cells Fractionated extracts of HeLa cells grown in tissue culture incorporate the deoxynucleotide units of deoxynucleoside triphosphates into a fraction which has the gross chemical properties of DNA and is rendered acid soluble by the action of pancreatic DNAase

The deoxynucleoside *di*phosphates cannot replace the *tri*phosphates In the experiment shown in Table 4, the labeled triphosphate is deoxyadenosine tri phosphate With the omission of thymidine triphosphate there is no detectable reaction and with the substitution of thymidine diphosphate, the system re mains inert Of interest, too is the fact that ATP even in very large amounts fails to replace deoxyadenosine triphosphate

What is the function of DNA in this reaction? In laboratory conversation we refer to it as a primer While the evidence for priming action is as yet indirect it is quite suggestive and we shall return to this point shortly What appears to

TABLE 3

REQUIREMENTS FOR TTP INCORPORATION INTO DNA

	mμm l
Complete system*	0 50
Omit C G A	00
Omit C	00
Omit G	00
Omit A	00
Omit Mg^{++}	00
Omit DNA	00
DNA pretreated with DNAase	00
Add ATP (100 mμmoles)	54
Add ADP (100 mμmoles)	0 50

5 mμm l f ea h deoxyn leos d triphosph t (TTP = 1 5 × 10⁶ p m /μm l) 1 m l f MgCl 3 γ f P ym 5 γ f DNA 30 mi tes t 37 C G d A t d f th triphosph tes f deoxy yt di deoxygu os d deoxy d nos espect ly

TABLE 4

INABILITY OF THYMIDINE DIPHOSPHATE TO REPLACE THYMIDINE TRIPHOSPHATE

	m m l DNA P
Complete system*	0 59
No TTP	00
TTP replaced by TDP	0 00

Th l b led l trat deo ARP PP

be an additional function of DNA is that of stabilizing the enzyme For ex ample when the purified enzyme was incubated under assay conditions in the absence of DNA followed later by the addition of DNA only 13 per cent of the activity remained Such an incubation in the presence of DNA resulted in a preservation of 87 per cent of the activity Ribonucleic acid obtained from to bacco yellow mosaic virus is inert in this respect as is acid treated DNA DNA partially denatured by heating or by alkaline treatment is less active than the native DNA in protecting the enzyme We had observed in the course of purifi cation procedures that the enzyme activity was found in a DNA nucleoprotein

tion The isolated DNA was then digested with pancreatic DNAase and chromatographed on a Dowex 1 column according to Sinsheimer s procedure (16) As described by him the products of the digestion consisted of small amounts of mononucleotides (about 1 per cent) and substantial quantities of dinucleotides (about 15 per cent) with the remainder of the digest present as higher oligonucleotides which in this case presumably were not eluted from the column In Figure 11 we see in the dinucleotide area of the chromatogram two

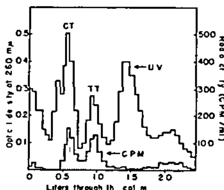


FIG 11 —Chromatographic distribution of dinucleotides in a digest of DNA into which C[14] labeled thymidine triphosphate had been incorporated

TABLE

IDENTIFICATION OF A DINUCLEOTIDE AS DEOXYCYTIDYLATE C[14] THYMIDYLATE

	μM les ml
Total I	1 30
I after phosphodiesterase	0 09
I after 5 nucleotidase	0 07
I after semen phosphomonoesterase	0 66
P after phosphodiesterase+5 nucleotidase	1 27
P after phosphodiesterase+semen phosphatase	1 33
Deoxycytidylate (after diesterase)	0 69
Thymidylate (after diesterase)	0 72

zones which judged by their spectral characteristics were considered to be a deoxycytidylate thymidylate dinucleotide and a dithymidylate dinucleotide The radioactive zones are indicated by the shaded areas Each of these dinucleotide zones was rechromatographed and then analyzed by chemical and enzymatic means The data shown in Table 7 are typical of what might be expected from a deoxycytidylate thymidylate dinucleotide The sample had 1 3 μmoles of phosphate all organic Essentially none of the phosphate was removed by 5 nucleotidase or by diesterase Fifty per cent of the phosphate was

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What is the function of DNA in this reaction? In laboratory conversation we refer to it as a 'primer' While the evidence for priming action is as yet indirect it is quite suggestive, and we shall return to this point shortly What appears to

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REQUIREMENTS FOR TTP INCORPORATION INTO DNA

	mμmoles
Complete system*	0 50
Omit C G A	00
Omit C	00
Omit G	00
Omit A	00
Omit Mg^{++}	00
Omit DNA	00
DNA pretreated with DNAase	00
Add ATP (100 mμmoles)	54
Add ADP (100 mμmoles)	0 50

* 5 mμmoles of each deoxynucleoside triphosphate (TTP — 1 5 × 10⁶ cpm per μmole), 1 μmole of $MgCl_2$, 3 γ of P enzyme, 5 γ of DNA, 30 minutes at 37° C. C, G and A stand for the triphosphates of deoxycytidine, deoxyguanosine and deoxyadenosine respectively

TABLE 4

INABILITY OF THYMIDINE DIPHOSPHATE TO REPLACE THYMIDINE TRIPHOSPHATE

	mμmol DNA-P
Complete system*	0 59
No TTP	00
TTP replaced by TDP	0 00

* The labeled substrate was deoxy ARP PP

be an additional function of DNA is that of stabilizing the enzyme For example when the purified enzyme was incubated under assay conditions in the absence of DNA followed later by the addition of DNA only 13 per cent of the activity remained Such an incubation in the presence of DNA resulted in a preservation of 87 per cent of the activity Ribonucleic acid obtained from tobacco yellow mosaic virus is inert in this respect as is acid treated DNA DNA partially denatured by heating or by alkaline treatment is less active than the native DNA in protecting the enzyme We had observed in the course of purification procedures that the enzyme activity was found in a DNA nucleoprotein

fraction and our attempts to separate the protein by chemical means failed to yield an active enzyme Here we observe that the enzyme now resolved from its native DNA by DNAase treatment is rather unstable and that only DNA among a group of materials tested can stabilize it

The specific and essential part played by DNA in the reaction itself is shown in Table 5 It is apparent here as in Table 3 that no reaction is detectable unless DNA is present Ribonucleic acid cannot substitute nor can albumin or acid treated DNA A reduced amount of activity is observed when alkali treated or mildly heated DNA is used in place of native DNA Control studies have shown that those substances which failed to act as primers do not inhibit the reaction upon the later addition of DNA The absolute requirement for DNA for the reaction was demonstrable not only by measuring the synthesis of DNA directly but also by observing (in an experiment to be described later) that DNA is essential for any detectable removal of the nucleotide residue of a triphosphate Thus there is an indispensable and highly specific function of

TABLE 5

SPECIFICITY OF DNA AS PRIMER

Addition	mμmoles DNA-P
None	0 00
DNA	69
Heated DNA	59
Alkali treated DNA	30
Acid treated DNA	00
RNA	0 00

DNA in this reaction which we are provisionally regarding to be that of a primer

What are the products of the reaction? Because all the measurements described up to this point were made with microscale incubation mixtures and at the tracer level of detection we should like to show that by working on a larger scale DNA synthesis can be detected by chemical methods But first it should be pointed out that our best preparations of enzyme still contain a disturbing amount of nucleolytic activities (and possibly other enzymatic activities) which confuse and plague any attempts to carry out refined studies of the reaction Digressing for a moment mention might be made of one such activity which until its removal in a late step of the purification resulted in rapid destruction of one of the substrates namely deoxyguanosine triphosphate As shown in Figure 10 this enzyme carries out a unique type of cleavage of this nucleotide yielding stoichiometric amounts of deoxyguanosine and inorganic triphosphate

Returning to the question of the net synthesis of DNA we have observed (Table 6) that there is an increase in UV absorbing material and in deoxypentose in the acid insoluble DNA fraction in amounts which are in fair agree

ment with the amount of isotope incorporated into this fraction In this experiment a comparison has been made with a control sample incubated under identical conditions except that one of the triphosphates has been omitted In such experiments the amount of DNA present at the end of an incubation period may be 50 per cent or so greater than that found in the control It should be emphasized however, that the synthesis observed in this experiment is a result of a balance between the synthetic and destructive activities in the preparation When the comparison is made with another type of control that is one in which the enzyme preparation has been omitted or one in which no incubation

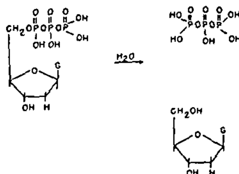


Fig 10 —Cleavage of deoxyguanosine triphosphate

TABLE 6

NET SYNTHESIS OF DNA

(in mμmoles)

	Control	Expt	Δ
P^{32} ×4	2	45	43
UV	77	115	38
Deoxypentose×2	55	91	36

Deoxy d os n triphosph t mitted

has taken place and in which as a result the added DNA was not destroyed the amount of synthesis while always detectable has not exceeded 10 per cent It is apparent from these results that no significance can be attached to the amount of net synthesis observed as long as the presence of nucleolytic activities complicates the picture

Regarding the chemical structure of the synthesized DNA we have been able to show that when a given deoxynucleoside tripho phate is incorporated into DNA it is linked by the 3 5 phosphodiester bridge which is characteristic of DNA In the following experiment thymidine triphosphate labeled with C^{14} in the C2 of thymine was converted to DNA under typical assay conditions after which large amounts of thymus DNA were added as carrier for its isola

tion The isolated DNA was then digested with pancreatic DNAase and chromatographed on a Dowex 1 column according to Sinsheimer's procedure (16) As described by him the products of the digestion consisted of small amounts of mononucleotides (about 1 per cent) and substantial quantities of dinucleotides (about 15 per cent), with the remainder of the digest present as higher oligonucleotides which in this case presumably were not eluted from the column In Figure 11 we see in the dinucleotide area of the chromatogram two

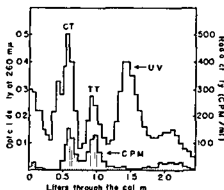


FIG 11 —Chromatographic distribution of dinucleotides in a digest of DNA into which C^{14} labeled thymidine triphosphate had been incorporated

TABLE 7

IDENTIFICATION OF A DINUCLEOTIDE AS DEOXYCYTIDYLATE C^{14} THYMIDYLATE

	μMoles ml
Total P	1.30
P after phosphodiesterase	0.09
P after 5 nucleotidase	0.07
P after semen phosphomonoesterase	0.66
P after phosphodiesterase+5 nucleotidase	1.27
P after phosphodiesterase+semen phosphatase	1.33
Deoxycytidylate (after diesterase)	0.69
Thymidylate (after diesterase)	0.72

zones which judged by their spectral characteristics were considered to be a deoxycytidylate thymidylate dinucleotide and a dithymidylate dinucleotide The radioactive zones are indicated by the shaded areas Each of these dinucleotide zones was rechromatographed and then analyzed by chemical and enzymatic means The data shown in Table 7 are typical of what might be expected from a deoxycytidylate thymidylate dinucleotide The sample had 1.3 μmoles of phosphate all organic Essentially none of the phosphate was removed by 5 nucleotidase or by diesterase Fifty per cent of the phosphate was

split by semen monoesterase and all of it was liberated by nucleotidase or monoesterase acting in combination with diesterase Diesterase alone produced deoxycytidine 5′ phosphate and thymidine 5 phosphate identified chromato graphically and spectrally Studies of the dithymidylate zone yielded quite analogous results

Physical studies of the DNA product have just been started with the gener ous assistance of Dr H K Schachman at the University of California (Berke ley) The curves shown in Figure 12 are the sedimentation distributions of a sample of thymus DNA without incubation and of samples isolated from experi mental and control mixtures (15) It is apparent that incubation of the DNA (control curve) has resulted in a greater heterogeneity of the DNA presumably

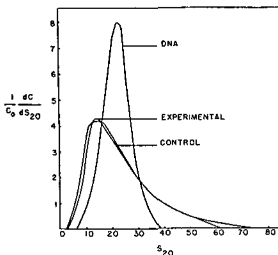


FIG 12 —Sedimentation distributions of DNA

as a result of nuclease action but it is also apparent that the sedimentation behavior of the DNA isolated from the experimental sample in which approxi mately 40 per cent of the nucleotides are newly synthesized as judged by its isotopic content is not significantly different from the control sample While a more precise description of the enzymatic product must await future work it is apparent from these physical studies that the newly synthesized DNA is a high molecular weight material similar to naturally occurring DNA On the basis of data presented thus far regarding this reaction it has been formulated in a provisional way as shown in Figure 13 In the presence of the four deoxy nucleoside triphosphates and DNA a condensation of nucleotidyl residues takes takes place to yield a polymer very similar by chemical and physical criteria to native DNA This condensation of the nucleotidyl residues should release equi

molar amounts of inorganic pyrophosphate, as is true of such condensations found earlier in coenzyme synthesis As is shown in Table 8 the incorporation of 20 mμmoles of deoxynucleotide into DNA (calculated from the incorporation of C^{14} labeled thymine residues) was matched by the release of equal amounts of inorganic pyrophosphate isolated by ion exchange chromatography and accurately measured by its P^{32} label The release of a small amount of inorganic orthophosphate is an indication of a phosphatase in the enzyme preparation used

The release of pyrophosphate was the basis for an experimental demonstration mentioned earlier that a given deoxynucleoside triphosphate is not acted

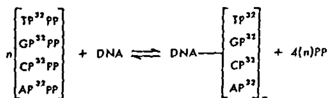


Fig 13

TABLE 8

Pyrophosphate Release from TTP
(in mμmoles)

	Control	Expt.	Δ
DNA C^{14}	1	21	20
P_i	8	12	4
PP_i^{32}	2	21	19

Deoxyad nos e tr phosph t mitted TTP w s l beled th C thym e d P th te m nal nd m d ll phosphate groups P d PP w re separated nd d term n d by ch mat gr phy on Do e l hl rid c l m

upon unless the complete reaction mixture is present In this case the release of radioactivity from thymidine triphosphate labeled in the terminal pyrophosphate group was absolutely dependent on the presence of DNA and the other three deoxynucleoside triphosphates

Evidence for reversal of this reaction may be seen in Table 9 where it is shown that the incorporation of inorganic pyrophosphate into deoxynucleoside triphosphates is dependent on the presence of DNA The estimation of this reaction is made here from the radioactivity originating in inorganic pyrophosphate which has been incorporated into an acid soluble Norit adsorbable fraction Such a reaction is not observed when inorganic orthophosphate replaces pyrophosphate The slight but nonetheless significant amount of reaction

found when the triphosphates are omitted suggests a direct pyrophosphorolysis of the added DNA It is important to note that the level of inorganic pyrophosphate necessary for these incorporations is about a hundred times greater than the levels of triphosphates used in the synthetic reaction Thus it appears likely that under physiological conditions little if any pyrophosphorolysis of DNA can be expected

In the work presented in this discussion an observation that has stood out most impressively is the absolute need for all four of the commonly occurring deoxynucleoside triphosphates (in addition to the need for DNA) for any detectable reaction However on a closer examination of this phenomenon we can

TABLE 9

APPARENT REVERSAL OF REACTION

	mμm les f P-DN l os d T ph ph t
Complete system	4 35
Omit enzyme	0 04
Omit DNA	0 09
Omit triphosphate	0 29
Replace P^{32} P^{1} with P^{3}	0 03

TABLE 10

INCORPORATION OF SINGLE NUCLEOTIDES INTO DNA

Add t	μμM les DNA P
C	2 5
C+G	5 1
C+G+T	15 7
C+G+T+A	3 300

C = deo ycyt d t ph ph te G = deo ygu nos t ph ph t T = thym d t phosph te A = deo y d t phosph t P labeled C t 1 7 2 × 10 ts pe m t pe μmol

really say no more than that omission of a single triphosphate reduces the level of synthesis to a value of less than 2 per cent of that of the complete system Translating this into the number of nucleotides that could be added to a chain of DNA (assuming the DNA molecules present in the reaction mixture to have molecular weights of 6 000 000) this would mean that the addition of about 20 nucleotides to each chain would not be detected We have therefore increased the specific radioactivity of our substrates by a hundred fold managing to keep the blank level of the reaction at its previous value The results of one such experiment are illustrated in Table 10 In this case the deoxycytidine triphosphate is labeled and with all the necessary components present 3 300 μμmoles

of deoxycytidylate nucleotide were incorporated into DNA. Omission of one of the triphosphates reduced the level of incorporation to about one half of 1 per cent of this value but even in the presence of only one triphosphate (and this has been observed with each of the other triphosphates as well) there is a small but significant and quantitatively reproducible incorporation of the nucleotide. Indications from numerous control experiments are that the omitted nucleotides are not being supplied as contaminants of the added nucleotides or as a result of liberation from the added DNA or from the enzyme preparation. It appears possible that we have with this highly sensitive technique the beginning of an approach to a more precise chemical definition of the synthesis of DNA by an enzyme system.

ADDENDUM.—Studies carried out since this paper was prepared provide additional information on this problem (J. Biol. Chem. 233, 159 1958; Proc. Nat. Acad. Sc. 44, 633 1958).

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CHAPTER 4

The Bearing of Structural Studies on Relationships between DNA and RNA

Alexander Rich

THERE are two classes of high molecular weight material inside cells which are capable of great specificity—the proteins and the nucleic acids Both are polymeric materials and it is generally assumed that their specificity arises from the particular sequence of their constituent residues as well as from their geometrical configuration A great deal has been known about proteins for many years but it is only comparatively recently that attention has been directed toward the role of the nucleic acids in biochemical systems Knowledge of this role is still incomplete and often inferential in nature but it does provide us with a background for studying the structural stereochemistry of these materials

Two kinds of nucleic acids are known and they are most easily differentiated on the basis of their chemical composition They are both polymers of nucleotide units which are themselves composed of a purine or pyrimidine base a sugar residue and a phosphate group In deoxyribose nucleic acid (DNA) the sugar is deoxyribose while in ribonucleic acid (RNA) it is ribose These differ by the presence of a hydroxyl group on C_2 of ribose Both nucleic acids have four types of bases—two purines and two pyrimidines—and three of these are common to both Thus DNA and RNA both contain the purines adenine (A) and guanine (G) plus the pyrimidine cytosine (C) However RNA has uracil (U) as the additional pyrimidine while DNA has 5 methyl uracil (thymine T) In brief these materials have a very similar composition with the exception of a systematic hydroxyl group for each nucleotide and a methyl group on one of the bases In some cases as in the bacteriophages additional residues are attached to the DNA bases These will be discussed elsewhere in this volume

The nucleotides of both DNA and RNA have been shown to be connected by the same linkages namely, through the phosphate group which is attached to the C_3 and the C_5' atoms of successive sugar residues In a schematic fashion, the polynucleotide chains for both DNA and RNA can be written as shown in Figure 1 However it should be noted that this chain is asymmetric, in that it

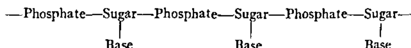


FIG 1

has a direction which is most easily seen by the sense of the C_3 to C_5 linkage of the sugar residues to which the phosphate group is attached

NUCLEIC ACIDS AND INFORMATION TRANSFER

DNA is found in the nucleus of cells and comprises one of the major components of the chromosomal material A great deal is known about the configuration of the DNA molecule It consists of two polynucleotide chains which are wound around each other in a helical manner with the nucleotides arranged so that the bases are hydrogen bonded to each other and the chains are held together by these hydrogen bonding pairs as first described by Watson and Crick (1) *The pairing arrangement is illustrated diagrammatically in Figure 2,* in which *A T G* and *C* stand for adenine thymine guanine and cytosine, respectively and *S* stands for the sugar deoxyribose and *P* for the phosphate group The pairing relation in this structure is such that adenine will pair only with thymine and guanine only with cytosine It should be noted that there are four possible types of base pairs as viewed in Figure 2 That is each of the two base pairs may be situated in two different ways Thus in a sense there are four different kinds of sites present located 3 4 A apart along the DNA molecule This distance is just the thickness of the purine or pyrimidine base The two backbone chains in DNA are arranged in an antiparallel fashion in the molecule One chain has its backbone rising, as shown by the arrows in Figure 2 while the other is descending

DNA is an extremely stable molecule It can be isolated from a large variety of tissues with its intricate hydrogen bonding system intact It is stable in this form in the absence of any other large molecules such as proteins and is usually prepared in the form of a salt

It is generally believed that DNA plays a major role in transmitting genetic information from one cell to the next The spermatazoon is largely a compact package of DNA which carries the entire genetic complement of one parent This genetic material has within it information necessary to reproduce the organism The information probably resides in the nucleotide sequence of DNA

The molecule is very long so that the permutations of arrangements of nucleotide bases could have enough specificity to completely define and differentiate one organism from another

During a cycle of cell division the DNA in the nucleus first increases twofold and is subsequently divided itself This results in an equal distribution of DNA through all the somatic cells of the organism (2) In addition to having the same amount of DNA it is believed that the molecules are qualitatively similar Duplication and division of this sort imply that the DNA molecule must be capable of undergoing molecular replication It has been suggested that the structure itself contains an indication of how this replicating process may be

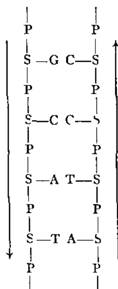


FIG 2

carried out (3) Each polynucleotide strand in the DNA molecule may serve as a template for replicating its complementary mate In this way one molecule will produce two molecules each of which is identical with the original molecule in terms of nucleotide sequence Although this replicating scheme is still not established there are good indications that the chromosomal material does act like a duplex structure (4) as would be required by such a mechanism

DNA is usually found associated with a variety of basic proteins Nonetheless there are good indications that the DNA alone carries the chemical information n cessary for reproduction In the bacteriophage system it has been shown that the DNA alone is injected into the bacterial cell during infection (5) In a similar way a transforming factor has been isolated from various bacterial

cells which has the power of changing some inheritable characteristics of the bacteria. This transforming factor has been found to be pure DNA.

However, it is apparent that the DNA molecule must do something more than simply replicate itself and be passed on from cell to cell. Somehow, DNA must influence and direct the metabolism of cells in such a way as to produce the specific molecular species which characterize the cells. How such metabolic activity is carried out by DNA is unknown at present. However, a great many indirect results have pointed to the suggestion that the DNA in some way synthesizes or influences the manufacture of an RNA molecule. The RNA molecule in turn is believed to carry out the metabolic role which is implicit in the genetic activity of DNA.

STRUCTURE AND FUNCTION OF RNA

In comparison to DNA, less is known about the structure and metabolism of the RNA molecule. RNA is widely distributed in both the nucleus and the cytoplasm. In the cytoplasm a large part of the RNA is associated with small compact particles approximately 180 A in diameter. Since these are found in the microsomal fraction during differential centrifugation they are called the microsomal particles. Additional RNA is found free in the supernatant. However, the configuration of the molecule in these sites is unknown.

It has recently been learned that RNA can function as a carrier of genetic information. This has been strikingly demonstrated with the tobacco mosaic virus (TMV), where the RNA in the virus has a role very similar to the DNA present in the bacteriophage particle. RNA isolated from TMV has an infectivity which exists in the absence of virus protein (6). Here again, as with the bacteriophage DNA, the RNA of the tobacco mosaic virus carries in it all the information necessary to replicate both viral RNA and protein. A similar finding has been reported with the mengo encephalitis virus. RNA isolated from cells containing the virus is able to produce an infection in the absence of protein (7). The implication of both these experiments is that the RNA molecule must undergo a replicating cycle.

In recent years a large number of biochemical experiments have directed our attention to the role which the microsomal particles play in protein synthesis. It has been shown that in liver cells all the protein synthetic activity is carried out by the microsomal particles (8). These particles are composed of roughly 50 per cent RNA and 50 per cent protein, and protein synthesis is strictly dependent upon the integrity of the RNA. It is commonly believed that the RNA molecule carries in the sequence of its bases some, or perhaps all, of the information necessary to carry out the synthesis of individual protein molecules.

Thus one of the common views regarding the over-all flow of chemical infor

mation in a cell is roughly the following The DNA molecule has in it most or all of the informational content necessary to carry out the specific molecular syntheses required to produce the entire cell This specificity is inherent in the sequence of nucleotide units in the molecule The DNA is transmitted from one cell to the next in the chromosomal apparatus In a daughter cell it proceeds to make an RNA molecule which has specificity in its base sequence In turn the RNA molecule may possibly undergo replicative cycles to produce more RNA after which it is bound into the protein synthesizing microsomal particles At this site specific protein synthesis occurs This picture of the transfer of chemical information through the cell from one macromolecular species to an other is somewhat speculative and most certainly oversimplified but it serves a useful purpose in directing research efforts It raises three major questions

1 How is the RNA molecule synthesized so that its base sequence reflects in some way the sequence specificity found in the DNA molecule?

2 How does the RNA molecule replicate? That this phenomenon must occur is clear from the experiments on tobacco mosaic virus

3 How does the RNA molecule transfer sequence specificity from itself to the protein molecules which are manufactured by the microsomal particles?

In a sense the last question is the broadest in that we are asking how genetic information finally becomes translated into the specific protein molecules which act as enzymes and structural units and make a cell capable of carrying out specific activities

There are no final answers to any of these questions as yet Part of the infor mation that is needed to obtain such answers probably will come from the de termination of the configuration and molecular structure of the RNA molecule as it carries out these various functions in the cell Just as the structure of DNA reflects the duplex character of chromosomal material and suggests a replication mechanism so it is likely that the determination of the structure of RNA will also suggest molecular mechanisms

Structural studies on RNA have not been very promising recently RNA can be isolated from tissues and is believed to exist in the form of an unbranched polynucleotide chain X ray diffraction studies have been carried out on oriented fibers of RNA which were prepared from a variety of sources (9 10) These all produce the same diffraction pattern (Fig 3 *a*) While this diffraction pattern is not well resolved into discrete reflections nonetheless it is sufficiently characteristic to say that all the material isolated from plant animal bacterial and viral sources has a common underlying configuration In the diffraction pattern there are strong meridional reflections at 3 3 and 4 0 A These would be expected if the planar purine and pyrimidine rings in the molecule were oriented at right angles to the fiber axis as in DNA Such an observation is compatible with the observed negative birefringence of these fibers In addition

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The fact that the diffraction pattern is not well resolved suggests that these preparations do not have their nucleotide units in a well ordered array. The material as isolated may represent a more or less stable but somewhat irregular helical coiling of polynucleotide molecules. It should be noted that RNA

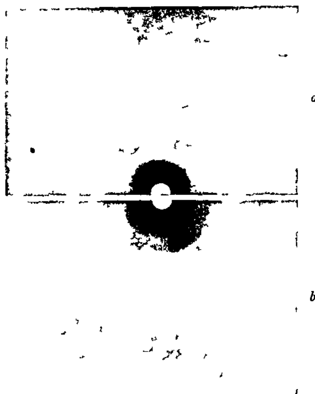


FIG 3.—Comparison of X ray diffraction photographs *a* Tobacco mosaic virus RNA *b* mixed polynucleotide polymer containing adenylic acid and uridylic acid residues (*AU*). The fiber axis is vertical, relative humidity is 80 per cent, CuK radiation. It can be seen that the two diffraction photographs are similar.

is somewhat unstable chemically and most extraction procedures probably disrupt the molecular configuration considerably.

Structural studies on RNA were greatly augmented by the discovery of an enzyme which would polymerize nucleotide diphosphate units into polymers which have all the characteristics of RNA (11). These synthetic polynucleotides have the same type of 3′ 5′ linkage as is found in the naturally occurring nucleic acid. In addition they have the same susceptibility to enzymatic diges

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The synthetic polyribonucleotides are important because they make it possible to study the configurational potentialities which are inherent in the RNA backbone Thus for example polymers can be made which have only one purine or pyrimidine base By studying the structures which various polynucleotides assume it is possible to determine how various bases affect the chain configuration Of special interest are the hydrogen bonding potentialities between bases since these are so important in maintaining the integrity of the DNA molecule Accordingly X ray diffraction studies have been carried out on a variety of polynucleotides in order to determine their molecular structure

THE STRUCTURE OF SYNTHETIC POLYRIBONUCLEOTIDES

Polyadenylic Acid

Polyadenylic acid (poly A) was the first synthetic polyribonucleotide to produce an oriented diffraction pattern This is illustrated in Figure 4 When allowed to dry from a concentrated solution poly A becomes sticky and can be pulled into oriented fibers with a strong negative birefringence ($\Delta n = -0\ 10$) The diffraction pattern is dominated by a very intense meridional reflection at a spacing of 3 8 A There is also a layer line spacing of 15 2 A which is fairly weak near the meridian but stronger farther out The birefringence of nucleic acids is due largely to the orientation of the unsaturated purine or pyrimidine rings These bases have π electrons which are easily polarizable in the plane of the ring and stacking of the rings in a direction at right angles to the fiber axis will produce negative birefringence The strong 3 8 A spacing on the meridian is therefore associated with adenine rings oriented more or less at right angles to the fiber axis However since the thickness of the purine ring is only 3 4 A there must be some structural detail in the molecule which keeps the rings from being flat on top of each other The absence of meridional reflections up to 3 8 A is characteristic of the diffraction pattern from a helical molecule A variety of models has been built for this molecule and one has finally been found which predicts the diffraction pattern (14) This is a two stranded helical molecule in which the adenine residues are hydrogen bonded to each other as shown in Figure 5 Two adenine rings are hydrogen bonded through the amino group and the ring nitrogen of the imidazole part of adenine This pairing of

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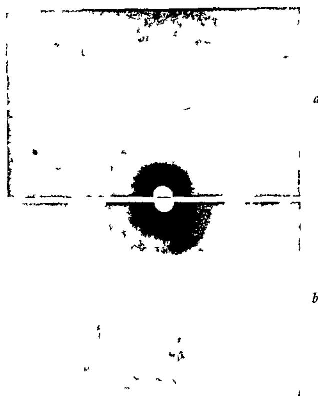


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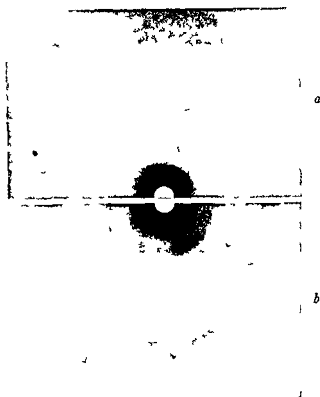


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adenine rings is identical with that which is found in adenine hydrochloride (15) In addition the ribose phosphate backbone is so arranged that an oxygen of the phosphate group from the opposite chain hydrogen bonds to the other hydrogen of the adenine amino group In this way the molecule is held together by four hydrogen bonds per base pair

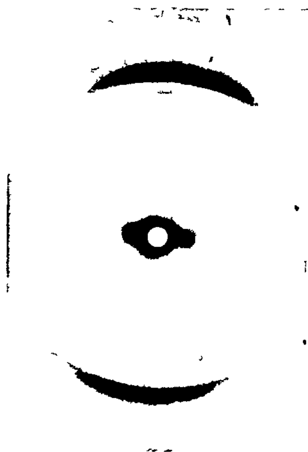


FIG 4—X ray diffraction photograph of polyadenylic acid The fiber axis is vertical, relative humidity 80 per cent The strong reflection on the meridian is at 3 8 A

Each chain in polyadenylic acid goes around a complete turn of the helix in 30 4 A However in this molecule the chains are parallel to each other That is the direction of C_3 and C_5 linkages in the two ribose phosphate chains is identical in marked contrast to DNA in which the backbone chains have antiparallel orientation Such an arrangement is shown schematically in Figure 6 where the two bands represent the ribose phosphate chain and the bars represent the hydrogen bonded adenine groups

Recently some work has been done on studying the stability of this molecule as a function of pH (16) It has been found that the pK of adenine has been shifted from 4 5 to a value of 6 5 in polyadenylic acid In addition the molecule is stable in the two stranded helical form only at pH s below 6 5 while at higher pH s the chains come apart and the molecule has the form of a random coil Hence another element in stabilizing the molecule is a proton which is found on the adenine ring In adenine hydrochloride it has been established that this proton is found on the N_1 of the ring and it is probable that it is there

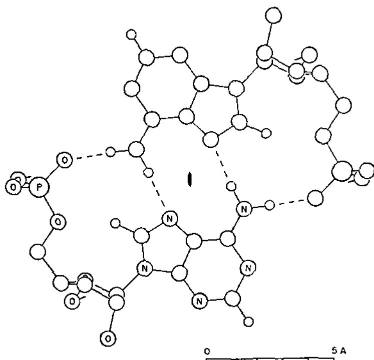


FIG 5—The arrangement of hydrogen bonds between the two chains in polyadenylic acid The view is along the fiber axis which has a twofold rotation axis Dashed lines are hydrogen bonds

also on the adenine residue of polyadenylic acid In this position the positive charge would be neutralized by the negative charge on the phosphate group of the opposite chain which is near the adenine ring at that point (Fig 5)

When this model is carefully built it becomes apparent why the adenine bases are 3 8 A apart along the helical axis—because of the hydrogen bonding between the phosphate group and the amino group of adenine The presence of the phosphate group in that position tilts the planar adenine residue above it in such a way that the spacing between the molecules remains at 3 4 A even though the translation along the axis is 3 8 A That is the adenine purine rings are tilted about 12 –14 from the normal to the fiber axis It is instructive to

It is quite remarkable that a mixture of two polyelectrolyte molecules both with identical charges will interact so specifically to make a regular structure like poly A + poly U It should be noted that this interaction occurs only at pH > 6 5 i e when poly A is in the form of a random coil At lower pH values no interaction occurs presumably because the two-stranded poly A is more stable We can learn something about the mode of combination by studying

TABLE 1

CHARACTERISTICS OF DNA AND OF POLY A + POLY U MOLECULE
(AT 78 PER CENT RELATIVE HUMIDITY)

	DNA	Poly A+ Poly U
Helical pitch of molecule	34 A	34 5 A
Residues turn	10	10
Distance between molecules in fiber	22 8 A	28 8 A
Birefringence of fibers	$\Delta n = -0\ 10$	$\Delta n = -0\ 10$
Intensity of diffraction pattern		
1st layer line	Medium	Very stron
2d layer line	Very strong	Medium

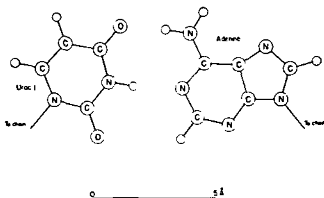


FIG 7 —The hydrogen bonding between adenine and uracil bases in polyadenylic acid + polyuridylic acid Hydrogen bonds are dashed lines.

the ultraviolet spectrum of this complex Both poly A and poly U have a maximum in their ultraviolet absorption spectrum at 259 mμ However when they are combined there is an increased hypochromic effect i e the resultant optical density of the complex is less than the sum of the component optical densities of the two polynucleotides In Figure 8 the dashed line shows the optical density of various mixtures of poly A and poly U keeping the total nucleotide concentration constant It can be seen that there is a sharp drop in

optical density which has a maximum at the 50 per cent point i.e. a 1:1 mixture of poly A and poly U. The sharpness of the drop at the mid point reflects the tightness of the binding. In this case the equilibrium constant for the reaction is large in the forward direction. By estimating the curvature at the mid point careful experiments have shown that over 98 per cent of the molecules are combined in the 1:1 mixture.

It has been shown that the combination of poly A and poly U is reversible. If the concentration of ions in the medium is decreased to less than 10⁻⁴ molar the two molecular species come apart. Thus an ionic strength of approximately 10^{-4} molar of uni-univalent salts is necessary to shield the considerable inter-

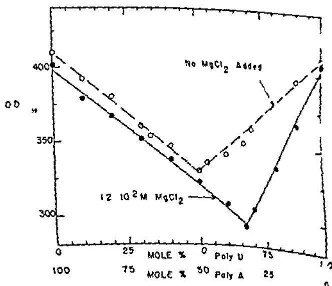


Fig. 8.—The optical density of various mixtures of polyadenylic acid and polyuridylic acid. Densities were measured 2 hours after mixing. All solutions are in 0.1 *M* NaCl, 0.01 *M* glycylglycine buffer, pH 7.4. *T* = 25° C.

molecular repulsive electrostatic forces. If the two molecular species are mixed together in concentrations of NaCl near 0.1 molar, the reaction velocity is very great and the reaction is complete in a very few seconds.

These experiments with mixtures of polyadenylic acid and polyuridylic acid demonstrate the stability of the DNA configuration. These experiments clearly show that it is possible for the RNA molecule to assume the form of a two stranded complementary duplex of the DNA type. The work described above suggests that RNA carries out molecular replication as does DNA; hence it is highly probable that it uses the same mechanism. If this should indeed be the case it simplifies the problem of understanding one phase of RNA metabolism.

Three stranded Molecules Polyadenylic Acid plus Two Polyuridylic Acid

In the previous section we pointed out the similarity between the structure of DNA and the complex of polyadenylic acid plus polyuridylic acid In a certain sense the complex is a simplified version of DNA in which a single base pair -adenine and uracil—exists at every interval of 3 4 A along the helix, whereas in DNA there are two different types of base pairing possible Because of this simplicity the poly A + poly U complex is an ideal model substance with which to illustrate chemical reactivities which may be properties of DNA itself In this section one of these chemical reactions is described

In Figure 8 (*dashed line*) the optical density of various mixtures of poly A and poly U is shown in 0 1 *M* sodium chloride at neutral pH If these measurements are repeated in the same system but modified by the addition of $1\ 2 \times 10^{-}$ *M* magnesium chloride the optical density-composition curve changes to the solid curve as shown in Figure 8 (21) This curve has a very sharp minimum at 67 per cent U and 33 per cent A i e a complex between two molecules of poly U and one of poly A This suggests that the poly A + poly U complex molecule is able to take on a third strand and become a three stranded molecule if there is a sufficient number of magnesium ions present in the medium

The change from the dashed curve of Figure 8 to the solid curve is accomplished in roughly 3 minutes with 10^{-2} *M* MgCl If the added magnesium concentration is decreased the rate at which the dotted curve changes into the solid curve decreases appreciably and the extent of reaction as well as the speed can be controlled critically by the amount of magnesium ion present in the medium This reaction is not dependent solely upon magnesium however as a variety of divalent cations have been found capable of effecting this transition Indeed even at high concentrations of sodium chloride (0 5–1 0 *M*) the formation of a three stranded complex will occur

Ultracentrifuge studies of this reaction have shown that there is roughly a 50 per cent increase in sedimenting velocity which the three stranded molecule has relative to the two stranded one This is just the increase in sedimenting velocity which one would anticipate if water molecules were displaced from the helical groove of the poly A + poly U complex and it were filled with a third polynucleotide chain In this case the frictional forces governing the descent of the molecule in the medium would not be altered appreciably because the shape has not changed but the density increment of the molecule relative to the surrounding solution will increase by approximately 50 per cent by the addition of a third chain

It has been possible to obtain a diffraction photograph of material prepared under conditions in which the three stranded complex (poly A + 2 poly U) forms The diffraction photograph is somewhat different from that produced by the poly A + poly U fiber A marked difference can be seen in the relative

intensities of the first and second layer lines The addition of the third strand has produced an intensification of the second layer line relative to the first

Studies have been carried out on the role of various ionic species in forming this three stranded complex (20) Divalent cations are one hundred times more effective than univalent ions in bringing about the formation of poly A + 2 poly U In a solution with the ionic composition found in tissues the three stranded complex is about half formed that is the third chain is loosely attached to the two stranded molecule Under these same conditions the two tranded molecule is firmly tied together

The method of attaching the third chain has not been worked out in detail at the present time It is possible that the third strand of polyuridylic acid attaches itself to the poly A + poly U complex by forming two hydrogen bonds with the adenine residue (i e poly U oxygen 6 and nitrogen 1 to adenine amino nitrogen and nitrogen 7 respectively) A system of this type would lead to a stable molecule in which the additional uracil residues are stacked on top of one another and helically wound around the groove in the poly A + poly U molecule

It is interesting to note that although the poly A + poly U molecule reacts with poly U it will not react with polycytidylic acid or polyadenylic acid Hence the reaction has a degree of specificity

Considerable interest is attached to the fact that a three stranded polynucleotide molecule is stable and has some specificity in its interaction with other polynucleotides As mentioned in the introductory section the DNA molecule has four different types of base pairs at each point along the helix If each of these points were capable of forming specific hydrogen bonds with a different ribonucleotide molecule it might be possible to use the formation of such a three stranded complex as a mechanism for synthesizing an RNA molecule which has sequence specificity (22) This sequence specificity would arise through the interaction of the oncoming base with one of the four types of base pairs found at each point on the DNA helix Thus a possible intermediary in the manufacture of RNA would be a two stranded DNA molecule with a single stranded RNA wrapped about its helical groove After this molecule is formed it could then unwind itself and carry on its metabolic activity in the cell This is of course an attractive possibility but it will take a great deal of further experimentation before such a mechanism can be fully evaluated

Polyinosinic Acid plus Polyadenylic Acid

The discovery that polyadenylic acid would combine with polyuridylic acid stimulated investigation into other possible combinations among the synthetic polyribonucleotides The enzyme polynucleotide phosphorylase is able to polymerize a mixed co polymer containing guanine residues but for reasons

which are wholly obscure it is unable to make polyguanylic acid. This polymer would be of great interest since, by analogy with poly A + poly U, we could then investigate its ability to combine with polycytidylic acid.

However, while lacking this ability, the enzyme is nonetheless capable of polymerizing a closely related substance, which is inosinic acid. The purine in inosinic acid is hypoxanthine, which is simply deaminated guanine. Inosinic acid is not found in naturally occurring RNA, even though it is believed to be a metabolic precursor of both adenylic acid and guanylic acid. In view of the structural similarity between inosinic and guanylic acids, it was of great interest to discover that polyinosinic acid would combine with polyadenylic acid to

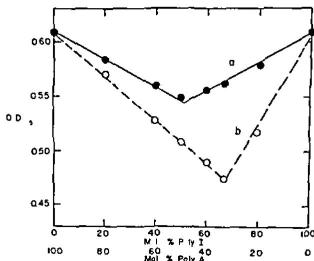


FIG. 9.—The optical density at 254 mμ of mixtures of polyinosinic acid (poly I) and polyadenylic acid (poly A). The solutions are both in 0.05 M NaCl, 0.001 M sodium cacodylate at pH 6.8, T = 25° C. All points have the same total concentration of nucleotide phosphate. The solid curve (*a*) is obtained at 31 minutes and the dashed curve (*b*) at 5 hours. The minimum shifts from 50 per cent poly I in *a* to 67 per cent poly I in *b*.

form a two stranded helical molecule (23). This combination can be observed by its absorption spectrum in the ultraviolet, by a study of the ultracentrifugal characteristics of the species, and through an X-ray diffraction study.

If polyinosinic acid (poly I) is mixed with polyadenylic acid in 0.05 M NaCl buffered to pH 6.8, the absorption spectrum of the mixture has a lower optical density than the average of the two individual species. Thus there is an increase in the hypochromic effect just as was observed for the system poly A + poly U. Equal concentrations of poly I and poly A have the same optical density at 254 mμ. If mixtures of these two species are made in 0.05 M NaCl near neutral pH, the optical density–composition curve appears as shown in Figure 9. At 31 minutes, the upper curve (*a*) has a sharp minimum at the mid

point indicating a 1 1 complex of poly A with poly I There is also an increase in the sedimentation constant when the complex is formed Under these conditions the poly I has an average S_{20} = 8 6 poly A has S_{20} = 7 2, and the 1 1 mixture has S_{20} = 10 4 at equilibrium If the ionic strength is decreased to near 0 01 *M* NaCl the curve remains as in Figure 9, *a* with a minimum at 1 1

An X ray diffraction photograph taken from a fiber with a 1 1 mixture of poly I and poly A is shown in Figure 10 This is a diffraction photograph which



FIG 10 —X ray diffraction photograph of a fiber of polyadenylic acid plus polyinosinic acid (1 1) The fiber is at a relative humidity of 66 per cent in a helium filled camera The fiber is tilted slightly from the vertical

is characteristic of a helix and is somewhat similar to a diffraction photograph obtained from the B form of DNA The helix has a pitch of 38 8 A and has about $11\frac{1}{2}$ residues per turn It forms a hexagonal lattice with a = 24 4 A The strong reflections in the 3–4 A region are again due to the stacking of planar purine bases at right angles to the fiber axis thereby accounting for the observed high negative birefringence ($\Delta n = -0\ 09$)

It is believed that inosinic acid like guanine is in the keto form for the oxygen on carbon 6 of the purine (24) In view of this it is quite likely that the

two purine bases are hydrogen bonded as shown in Figure 11 To build the structure these base pairs are stacked helically with 31½° rotation between each base pair and the ribose phosphate chains on the outside Although Fourier transform studies have not yet been completed it is probable that the ribose phosphate chains are antiparallel on the outside of the molecule

Thus this combination is similar to DNA in some ways but strikingly different in that it represents the combination of two different purines to form a hydrogen bonded pair rather than a purine with a pyrimidine It is worth noting that guanine itself could form hydrogen bonds in this way with adenine since the amino group would not interfere with the arrangement shown in Figure 11

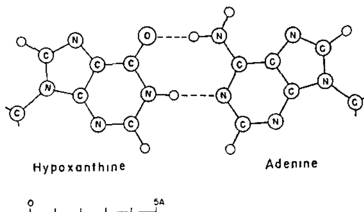


FIG 11 —The hydrogen bonding between hypoxanthine and adenine in polyinosinic acid + polyadenylic acid One carbon atom from the ribose ring is attached to each base

It is possible that such an adenine guanine pairing could be of importance in naturally occurring nucleic acid It has been suggested that an occasional replacement of guanine for thymine might occur in DNA (25) This could happen only if there were some distortion of the molecule at that point hence it would be a very infrequent occurrence However this might be a possible basis for a mutation The possibility of introducing an anomalous purine into DNA has been suggested by earlier work since a thymine requiring mutant of *Escherichia coli* has been found to contain 6 methyl amino purine (26) Apparently this purine appears to replace thymine rather than adenine as judged by analytical data since the molar quantity of thymine plus 6-methyl amino purine is equal to adenine

Three stranded Molecules Polyadenylic Acid plus Two Polyinosinic Acid

The analogy with the system poly A + poly U is further strengthened by the observation that the poly A + poly I molecule will take on an additional strand of poly I to become three stranded This can be shown most clearly by the

ultraviolet absorption spectrum Figure 12 shows the spectrum of equimolar solutions of poly I and poly A If a mixture is made with two thirds of poly I and one third of poly A the resultant spectrum is lower than either of the originals This change can also be seen in the optical density–composition curve (Fig 9) where the lower curve (*b*) is obtained at equilibrium in 0 05 *M* NaCl at pH 6 8 In that curve the minimum has shifted to 67 per cent poly I and 33 per cent poly A i e a 2 1 complex Ultracentrifugal analysis shows that the 2 1 complex has an even larger mean sedimentation constant than the 1 1 complex (S_0 = 14 7 for 2 1 and 10 4 for 1 1)

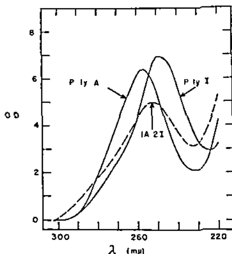


Fig 12 —*Solid lines* absorption spectra of polyadenylic acid (poly A) and polyinosinic acid (poly I) before mixing The solutions are both in 0 05 *M* NaCl 0 001 *M* sodium cacodylate at pH 6 8 *T* = 23 C *Dashed line* absorption spectrum of a 1 2 mixture (1A 2I) when equilibrium is reached As the complex forms there is a drop in the optical density

At the present time it is not known how the third chain is attached to the two stranded complex It could form two hydrogen bonds to the adenine residue as suggested for the second poly U chain in poly A + 2 poly U

The extent and velocity of the reaction is determined by the amount of electrolyte present in the medium In 0 1 *M* NaCl the second poly I strand joins the other two very quickly and the reaction is completed in less than 1 minute However in 0 02 *M* NaCl it may take as long as several hours to complete the formation of the three stranded complex

Polyinosinic plus Polycytidylic Acid

In view of the similarity between inosinic acid and guanylic acid mentioned above it is perhaps not surprising to find that polyinosinic acid combines with polycytidilic acid in a 1 1 complex (27) The formation of the complex can be

seen through an optical density–composition diagram similar to Figure 9 However in this case only one complex is observed with a sharp minimum at 50 per cent

X ray diffraction patterns have been obtained from these mixtures and they show a helical diffraction pattern which is not very well resolved as yet but which seems to resemble the RNA pattern seen in Figure 3 This pattern remains to be interpreted in terms of molecular configuration



FIG 13 —X ray diffraction photograph of a fiber of polyinosinic acid The fiber axis is vertical The strong reflection on the meridian is at 3 4 A

Polyinosinic Acid

Negatively birefringent fibers of poly I produce an oriented diffraction pattern (28) (Fig 13) This diffraction pattern is a very unusual one for a polynucleotide since the first layer line on it occurs at 9 8 A in contrast to the layer line spacings which occur at 30–40 A in DNA (poly A + poly U) or any of the other combinations described previously However the diffraction picture has a strong reflection on the meridian at 3 4 A which is undoubtedly produced by the stacking of the hypoxanthine bases in the molecule Careful measurements show that the diffraction photograph is one produced by a helical molecule Such a diagram can be interpreted by assuming that symmetry in the molecule cancels the layer lines at spacings greater than 9 8 A This

can best be accounted for by a three stranded helical molecule which has the hydrogen bonding shown in Figure 14 Here the hypoxanthine is in the keto form and the system has cyclic hydrogen bonding i e each strand receives a hydrogen bond from one strand and gives one to the other strand

The ribose phosphate chains in polyinosinic acid must be parallel to each other to produce this diffraction pattern and each chain goes around the molecule once in 29 4 A However, because the three chains in the molecule are related by a threefold symmetry axis the first two layer lines are canceled leaving the third layer line at 9 8 A

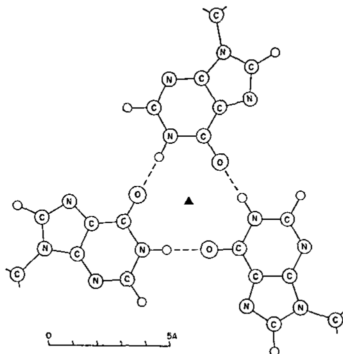


Fig 14 —The hydrogen bonding between three hypoxanthine bases in three stranded polyinosinic acid A threefold rotation axis is present

The formation of this complex is sensitive to the pH of the medium Thus if the pH is raised slightly and some of the hydrogen atoms are removed from the base the three stranded complex begins to break up This can be observed in the ultracentrifuge since the sedimentation constant of poly I decreases as the pH is raised The three stranded complex probably becomes unstable because the hypoxanthine group no longer has a proton on nitrogen 1 to use in the cyclic hydrogen bonding

Polyinosinic acid in this form has some features in common with poly

adenylic acid in that it is a multiple chain structure in which the ribose phosphate chains are all parallel and the purine bases systematically hydrogen bonded to each other

CONCLUSION

Our knowledge of the structure and function of the nucleic acids is quite incomplete We do not as yet have solutions to the fundamental problem of how molecular replication of RNA occurs or how RNA may be synthesized by DNA We do not know how an RNA molecule aids in determining the sequence specificity of protein However studies of the synthetic polyribonucleotides have been instructive in providing suggestive answers to at least some of the questions These studies have given rise to the suggestion that RNA may undergo molecular replication in a form very similar to DNA utilizing the complementary two stranded helix In addition the formation of three stranded molecules has suggested that such mechanisms may be important in the formation of RNA from a two stranded DNA molecule

In the field of protein structure the synthetic polypeptides have proved useful in aiding the solution of the structure of the fibrous proteins It is to be hoped that the synthetic polyribonucleotides will play a similar role in facilitating our understanding of the structure of ribonucleic acid and in helping to provide insight regarding the function of this molecule in biochemical systems (29)

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CHAPTER 5

Protein Structure and Biological Activity

Christian B Anfinsen

WE HAVE only recently reached that stage of methodological and conceptual sophistication where a consideration of the relationships between the structure of protein molecules and their biological activity has become possible Nevertheless the literature has already been adequately saturated with discussions of the subject, and, for that reason the following remarks have been restricted to the presentation of a thin background of experimental data together with a few conclusions and questions which might not be completely obvious to those who are not directly engaged in protein research or in allied fields

The fundamental work of Sanger and his collaborators (1, 2 3) followed by similar studies by a number of other groups of investigators (e g 4–12) has now given us a body of information on the covalent structure of proteins which permits us to proceed with the business of the systematic degradation of protein structure in parallel with studies of the effects of such modification on the functional capacity of these molecules A number of investigations have demonstrated that biologically active proteins may be considerably modified with only minimal loss of function Two examples of such work have been chosen here for more detailed chemical consideration

The structure of the adrenocorticotrophic hormone (ACTH) prepared from the pituitary glands of two different species has been completely elucidated in several laboratories (7 8 9) and recently the structures of allied melanocyte stimulating hormones (MSH) have also been worked out (10 11) The various structures derived from pig pituitary glands are listed in Figure 1 They are arranged in such a way as to make clear the overlapping portions of the amino acid sequences The figure also includes an indication of some of the sites at which these polypeptides are cleaved by the various proteolytic enzymes that were used during the course of structural study Although the full story is not

et available it appears that at least the eleven C terminally located residues of ACTH may be removed by digestion with pepsin without loss of hormonal activity (7) We may thus conclude that no more and perhaps less than the first twenty eight residues are sufficient On the other hand removal of one or two residues from the N terminal end of the molecule by leucine aminopeptidase digestion leads to complete inactivation Recent studies by White and Gross (13) have shown that bovine fibrinolysin which splits corticotropin following residues 8 and 15 also causes complete loss of ACTH activity Measurements of MSH activity (which one might expect to disappear during such hydrolysis) were unfortunately not reported The melanocyte stimulating activity of ACTH is however not lost following the more limited modification

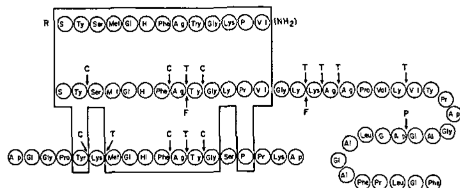


FIG 1 —The structures of some porcine pituitary hormones *Upper formula* α MSH (10) *middle* ACTH *lower* β MSH (10 11) *C* chymotrypsin *T* trypsin *F* fibrinolysin *P* pepsin N terminal groups at left

at the N terminal end of the chain brought about by leucine aminopeptidase (9)

Two different polypeptides having melanocyte stimulating properties but no ACTH activity have been isolated from pig pituitary glands The first termed β MSH contains eighteen amino acid residues and includes a sequence of seven residues which is found also in ACTH (10 11) The second α MSH contains only thirteen residues and the N terminal and C terminal ends are apparently masked by an acyl group and an amide group respectively (10) The sequence of this polypeptide is completely analogous to the N terminal portion of porcine ACTH except for the modifications at the ends of the chain just mentioned

Although it is perhaps superfluous to discuss here the activity interrelations summarized in Figure 1, since the matter has been considered in detail by Li *et al* (11) and by Harris and Roos (14) and Harris and Lerner (10) the implications of these findings in connection with the mechanism of polypeptide

biosynthesis are worthy of note The heptapeptide sequence which occurs in all three of the molecules shown is suggestive of a common intermediate in the synthesis of all three It might be argued that the same template is involved in the formation of β ACTH and α MSH since the structure of the latter is completely contained in the former excepting the terminal modifications although of course the existence of different templates having similar information is equally probable In the case of β MSH the former proposition becomes less tenable because the heptapeptide sequence is surrounded by residues not present in the other two cases The recent studies of Ramachandran and Winnick (14) on pituitary extracts indicating a very heterogeneous and large pool of assorted peptides would be compatible with a situation in which peptide fragments are first synthesized *de novo* or produced through controlled degradation of some preformed protein material and then subsequently assembled in a specific way to yield the various biologically active polypeptide materials which characterized the anterior lobe and the pars intermedia of the pituitary gland It may be argued that these polypeptides are small and that their synthesis may not parallel that of larger protein molecules Nevertheless, the structural data concerned here are of considerable interest in connection with the hypothesis that protein biosynthesis involves discrete kinetically distinguishable (15) intermediates whose specific alignment and conjugation is brought about by genetically controlled assembly mechanisms

A second example which may be considered in a preliminary sort of way is the enzyme bovine pancreatic ribonuclease (4–6) The structural elucidation of this protein has not yet been completed but sufficient is already known to permit us to make a few remarks about its unique characteristics A schematic summary of our present picture of the chemical formula of ribonuclease is shown in Figure 2 It must be emphasized that this scheme has all the deficiencies of a two-dimensional projection and that the spatial relationships between the various parts of the molecule as it exists in solution must be arrived at through a careful analysis of the physical studies which are now only in their beginning stage Ribonuclease is a single chain of 124 amino acid residues cross linked through 4 disulphide bridges and an undetermined number of non covalent bonds of varying strengths It has a fairly long N terminal 'tail' which according to the results of Richards (16) may be almost completely removed by a limited exposure to the proteolytic enzyme subtilisin without loss of catalytic activity in the macromolecular portion It also appears that the C terminal valine residue and possibly the preceding serine and alanine residues as well may be removed by carboxypeptidase action without impairment of activity (17)

The converse of these findings is obtained when one exposes ribonuclease to very limited digestion with pepsin (18) From such a digestion mixture one may isolate a derivative of the native molecule which lacks only the C terminal

tetrapeptide Asp Ala Ser Val as evidenced by the presence in this derivative of C terminal phenylalanine (19) and by the absence of indications of other cleavages in the polypeptide chain This derivative is completely *inactive* Concomitant with the covalent change there occurs a marked change in the ultra violet absorption of the tyrosine moieties of the protein, of a sort consistent with a process of hydrogen bond rupture in some tertiary structural feature of the molecule involving the hydroxyl groups of some of the tyrosine rings and electronegative groups elsewhere in the molecule (19) The simultaneity of the changes in activity and spectral properties and of the rate of appearance of

Fig 2 —Schematic diagram of the structure of bovine pancreatic ribonuclease *An* asparagine *Gn* glutamine The sequences of the residues that are crosshatched are not definitely determined at the present time This figure has been constructed from the combined studies of a number of individual at the Rockefeller Institute (including Drs C H W Hirs S Moore W Stein D Spackman and L Bailey) and at the National Heart Institute (including R Redfield J Cooke A Ryle and C B Anfinsen)

the free tetrapeptide is shown in Figures 3 and 4 This change induced by pepsin may be correlated with a more or less direct attack on the active center of the molecule and suggests that this catalytically active constellation of amino acid residues and three dimensional structural parameters is associated with the C terminal portion of the protein A similar conclusion may be reached from the recent degradative studies of Rogers and Kalnitsky (20)

It must be kept in mind however that cross linking through disulfide bridges may bring into close spatial relation two areas of the molecule quite widely separated in terms of amino acid sequence The locations of the disulfide bridges are indicated in Figure 2 (21 22) The half cystine residue located at position 40 in the chain is bonded to the half cystine residue at residue 113 that at 58 to 96 and so forth Thus C terminally located residues are

drawn into close proximity to portions of the sequence well separated from this end of the chain in a linear sense (model building although clearly of enormous difficulty in the case of a polypeptide of this size appears to be an unfortunate necessity for obtaining a better understanding of the three dimensional aspects of function in this enzyme)

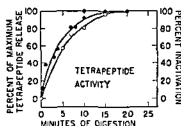


FIG 3 —Inactivation of ribonuclease and appearance of the tetrapeptide Asp Ala Ser Val during pepsin digestion at pH 1 8

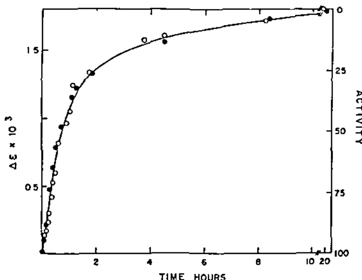


FIG 4 —Relation between spectral and activity changes during pepsin digestion of ribonuclease *Left ordinate* change in extinction at 285 mμ (*open circles*) *right ordinate* per cent of native activity (*solid circles*)

Recent studies on the stepwise reductive cleavage of the SS bridges of ribonuclease using thioglycollate (23) have been encouraging in connection with the likelihood of obtaining a really small fragment of the enzyme that still possesses enzymatic activity The experiments summarized in Figure 5 indicate that certainly one and perhaps two of the SS bridges may be so ruptured with only partial inactivation Furthermore it can be shown that the activity

which remains is not due to remnants of unattacked native enzyme, since electrophoretic analysis indicates the complete absence of the unchanged protein Although based on very preliminary data the conclusion can be drawn that one bridge which is *not* ruptured by the limited reduction process and which therefore is implicated in the active core of the molecule is the disulfide bond joining half cystines 3 and 7 (numbering from the N terminal end of the chain) which are located at residues 58 and 96 This bridge appears to be intact in the active products of partial reduction

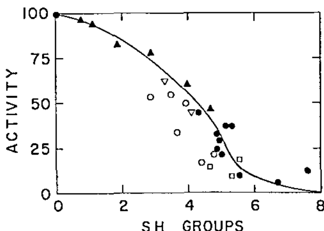


FIG 5—Changes in ribonuclease activity during reductive cleavage of disulfide bridges *solid triangles* reduction with thioglycollate in absence of urea *solid circles* in presence of 8 *M* urea. Open points represent results of reoxidation experiments open squares oxidation with gaseous oxygen of fully reduced (8 SH groups/mole) inactive ribonuclease other open points oxidation of partially active intermediates (Recent experiments indicate that the reduction of disulfide bridges in ribonuclease in the absence of urea does not proceed beyond two SH groups per mole and that the higher levels of reduction indicated by three of the solid triangles in the figure may be due to side reactions The shape of the curve is not changed however) For details see reference 23

These studies on the pituitary gland hormones and on ribonuclease have been chosen as examples from a number of investigations of permissible modifications (pepsin papain TMV lysozyme etc) They illustrate some generalities that may well be quite applicable to many biologically active proteins and that make it necessary for the protein chemist to expand his horizons and his research plans considerably A few of the questions raised are the following

1 If through the millions of years of mutation and selection nature has chosen and preserved certain unique molecular structures which appear to our naive mind s eye to contain superfluous parts are we not led to believe that a number of the structural features in proteins have to do with yet undiscovered aspects of cellular engineering and metabolism?

2 Is it therefore not likely that in analogy to the process of natural selec

tion at the morphological level, there exists a "natural selection of molecules with a number of different structural aspects within the same molecule each to be considered in the evaluation of a given molecular species in terms of its relative efficiency and suitability in the total cellular economy?

3 If relatively small portions of catalytically active polypetides are sufficient for the job of catalysis may one properly speculate about the possibilities of pre Cambrian organisms so simple and so well supplied with environmental food supplies that the advantages of macromolecularness were not yet an overriding consideration?

4 May we begin now to think of certain mutants such as the temperature dependent ones described by Hartmann (24) for some of the enzymes involved in histidine biosynthesis in *Salmonella* as containing modified genetic loci which control the biosynthesis of parts of proteins intermediate in functional importance but perhaps not absolutely obligatory as structural components? The relatively low frequency of occurrence of "temperature mutants" in a mixed mutant population may be a hint in this direction (see for example articles by Benzer [25])

Questions such as these as well as the whole problem of the significance of species variations in protein structure and the genetic basis for such variations, will clearly occupy investigators in many allied fields for a great number of years It does not seem too overoptimistic however to feel that the present degree of interest in the molecular basis of biological function will increase even further and that partial answers to at least some of these problems may be obtained through experimental study

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CHAPTER 6

The Kinetics of β-Galactosidase Induction*

Aaron Novick and Milton Weiner

A BACTERIAL population may acquire the capacity to use a new substrate in two ways. If there are in the population any mutant individuals able to utilize the new substrate, they will grow and give rise to a culture that is composed entirely of mutant type organisms. Alternatively, the bacteria initially present may adapt to the use of the new substrate when, for example, the new substrate acts as an "inducer," causing the bacteria to produce the enzyme(s) necessary for its utilization. In the first case the capacity to utilize the new substrate behaves like an inherited characteristic, since a mutant individual able to use the substrate gives rise to descendants also able to use it, whether the substrate is present or not. In the second case the capacity to use the substrate is apparently not inheritable, since the ability to produce the enzyme (the induced state) can be maintained only in the presence of the substrate.

Recently, however, an example has been observed in which the induced capacity to utilize a substrate simulates a genetic alteration in the sense that under identical culture conditions bacteria having the necessary enzymes give rise to descendants having them, while bacteria lacking the enzymes give rise to descendants lacking them (1).

These studies have been made in *Escherichia coli* bacteria with the inducible enzyme β-galactosidase, which is necessary for the utilization of the sugar lactose. There are some synthetic sugars, like thiomethyl-β-D-galactoside (TMG), that are potent inducers of β-galactosidase formation although they are not themselves hydrolyzed by this enzyme. For this reason, in all the experiments described below, TMG was used as the inducer.

It was discovered by Monod's group at the Institut Pasteur in Paris that

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both TMG and lactose induce not only β galactosidase but also a specific mechanism for the transport and concentration of inducer within the bacterium (2) This mechanism presumably enzymatic in character they have called galactoside permease, and it is distinct from β galactosidase

The presence of an inducible permease manifests itself in two ways In the first place bacteria containing permease can be shown to concentrate inducer within the cell to levels a hundred times that in the medium (2) In the second place the existence of an inducible permease enables one to understand the otherwise curious kinetics of induction of β galactosidase The present paper consists of a description of the kinetics of induction of β galactosidase and of a discussion of how these kinetics can be explained by the action of the inducible permease In addition some experiments are described which give further insight into the nature of permease The experimental techniques and some of the observations reported here have been described in a recent paper (1)

THE INDUCTION OF β GALACTOSIDASE FORMATION

In a typical induction experiment the inducer is added at the desired concentration to a culture of growing bacteria The concentration of enzyme E, calculated per bacterium rises with time to a constant ultimate value The rate at which the concentration of enzyme rises dE/dt is given by

$$\frac{dE}{dt} = aS - aE \tag{1}$$

where a is the bacterial growth rate constant and S is the average rate per generation at which the bacteria form β galactosidase The synthetic capacity of the culture S is therefore given by

$$S = \frac{1}{a}\frac{dE}{dt} + E \tag{2}$$

When a high concentration of TMG (5×10^{-4} M) is added to a growing culture of bacteria the rate per bacterium S at which β galactosidase is made reaches its maximum value in a few minutes As a result E reaches 50 per cent of its ultimate value after one bacterial doubling (63 per cent after one generation here defined as the time required for the bacterial population to increase e fold) An example of an experiment under these conditions is furnished in Figure 1

At lower concentrations of inducer, the kinetics of induction are curious in the sense that the rate of enzyme formation continues to rise for many generations after the addition of inducer The rate ultimately reaches a constant value (intermediate saturation) which for lower concentrations of indu[illegible] less than the maximum value S_m found at higher concentrations The [illegible] of a typical experiment are shown in Figure 2 where both the enzyme [illegible]

tration E and the computed rate of synthesis, S, are plotted as a function of time It should be noted that the rate of formation of enzyme, S, rises linearly from zero time until it approaches the constant saturation value In experiments using a range of concentrations of TMG it was found that with an increase in the concentration of inducer there is a sharp increase in the slope of the straight line and an increase in the ultimate saturation value As the concentration of TMG is increased, the saturation value increases until the maximum capacity (S_{max}) for making β galactosidase is reached

It can be shown, on the one hand that, at high concentrations of inducer such as those given in Figure 1, all the individual bacteria in the population

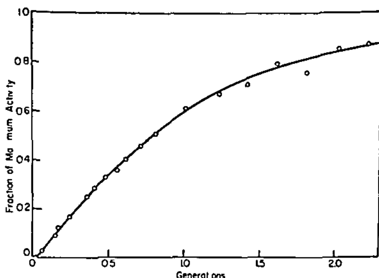


FIG 1 —Rise in β galactosidase activity following addition of 5×10^{-4} M TMG to a growing bacterial culture

make enzyme at an identical rate (3) On the other hand at low concentrations of inducer as in the experiment shown in Figure 2 the bacterial culture was found to consist of two kinds of cells those (induced) making β galactosidase at the maximum rate and the remainder (uninduced) making essentially none (1) The slow rise in the capacity of the culture to form enzyme is the result of a rise in the fraction of the bacteria in the induced state

The all or none character of the state of induction at low concentrations of inducer can be understood in terms of the functioning of the inducible concentrating mechanism galactoside permease When inducer is added to the culture permease synthesis is initiated The rate of formation of permease is determined by the concentration of inducer in the medium, but the rate may be so small at the lower concentrations of inducer that the probability of a

bacterium's making a single permease unit during its lifetime is small (It will be assumed that permease is an enzyme in the usual sense of the word and that the smallest unit is a single molecule) Once a bacterium has made a functioning permease molecule there will be an increase in concentration of inducer in the cell which in turn will increase the probability that a second permease molecule will be formed The presence of two permease molecules further increases the rate of permease formation and in this way there should be an autocatalytic rise in the permease content of the bacterium to some maximum value When a bacterium has maximum permease sufficient inducer is concentrated to enable the bacterium to synthesize β galactosidase at a high rate

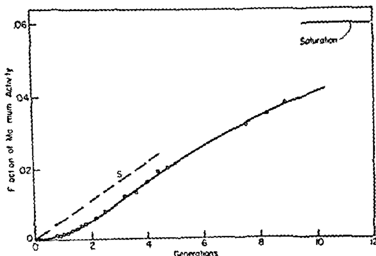


FIG 2 —Rise in β galactosidase activity following addition of 7×10^{-4} M TMG to a growing bacterial culture Broken line gives rise in S the computed synthetic rate

Moreover all the progeny of such a bacterium will have maximum amounts of permease as long as the inducer is present in the medium and as long as each daughter cell receives at least one permease molecule at the time of division

To become induced a bacterium must reach a threshold level of permease content which will permit the permease content to rise to maximum The fact that the linear rise in S begins from zero time suggests that the threshold here corresponds to a single permease molecule Were the threshold to be two or more molecules of permease a lag of one generation or more would be expected before the rise in the capacity for synthesis becomes linear On the basis of this picture the slope K of the linear rise in capacity to make β galactosidase is determined by the probability of the appearance of the first permease molecule

The rise in capacity to form enzyme often levels off at some intermediate

saturation value less than maximum e g at about 6 per cent in Figure 2 These intermediate saturation levels where the fraction of the population in the induced state is constant result from the fact that, in the presence of the inducer uninduced bacteria grow more rapidly than induced As a result a steady state level is reached where the number of bacteria becoming newly induced is canceled by an apparent loss of induced cells due to their slower growth The saturation value ultimately reached is given by either

$$\frac{S}{S_{\infty}} = \frac{K}{1 - (\alpha_1/\alpha)} \qquad \text{for } K + \alpha_1 < \alpha \qquad (3)$$

or

$$\frac{S}{S_{\infty}} = 1 \qquad \text{for } K + \alpha_1 \geq \alpha \qquad (4)$$

where α is the growth rate constant of the uninduced bacteria and α_1 is that of the induced bacteria (The difference in growth rate is about 7 per cent at lower inducer concentrations and increases as the concentration of inducer is increased)

These interpretations are consistent with the observation that there is a low concentration of inducer called the *maintenance concentration* that will indefinitely maintain the full synthetic activity of an initially fully induced culture whereas at this low concentration of inducer an initially uninduced bacterial culture never makes enzyme at more than a negligible fraction of the maximum rate In the case in which an initially uninduced culture is transferred to the maintenance concentration of inducer the fraction of the population in the induced state rises to a constant value (intermediate saturation) which is very small at this low concentration of inducer Alternatively if *all* the bacteria in a culture are initially induced by prior exposure to a high concentration of inducer all of them and their descendants will be maintained induced after transfer to the maintenance concentration of inducer In this case there are no uninduced bacteria that would "outgrow the induced ones, and, therefore the synthetic activity of the pre induced culture is maintained at the maximum value

The maintenance phenomenon can be used as the basis of a simple technique for determining the fraction of induced bacteria in a population Single bacteria are transferred to a maintenance concentration of inducer and grown to a population of a size (10^8 or greater) which permits easy β galactosidase assay An induced bacterium will give rise to a culture with maximum β galactosidase content while an uninduced bacterium will produce a culture of very low enzyme content

This technique was used to demonstrate that the linear rise in the capacity to form enzyme is the result of a rise in the fraction of the bacteria in the in

duced state Also it was shown in this way that at intermediate saturation the culture consists of a mixture of constant proportions of induced and uninduced bacteria In each case the rate of enzyme synthesis expressed as the fraction of maximum rate equals the fraction of the population that gives rise to cultures of maximum enzyme content when single cells are transferred to a maintenance concentration of inducer It can be argued therefore that for the individual bacterium at a concentration of inducer such as that shown in Figure 2 only a short time is required between the appearance of the first permease molecule and the achievement of maximum levels of enzyme production

Evidently the kinetics of β galactosidase induction at low concentrations of TMG resemble the kinetics of the accumulation of mutants in a population In both cases there is the accumulation at a constant rate of altered clones of bacteria—on the one hand clones having by induction acquired the capacity to produce β galactosidase and on the other hand clones having by mutation acquired some new character The analogy extends further, to the case of intermediate saturation which can be likened to the steady state when there is selection against the mutant phenotype

THE LOSS OF CAPACITY TO FORM β GALACTOSIDASE

If a population of fully induced bacteria is transferred to and grown in medium containing no inducer β galactosidase synthesis immediately ceases and the enzyme present is diluted out among the daughter bacteria Between zero and the maintenance concentration of inducer there is a range of concentrations at which the enzyme level is not maintained because of a progressive fall in the rate at which β galactosidase is made In this range of concentrations it was found as is shown in Figure 3 that the enzyme concentration falls exponentially with time at a rate determined by the inducer concentration

In experiments of the kind shown in Figure 3 in addition to the fall in the concentration of β galactosidase there must be a fall in the concentration of permease since the rate of synthesis of β galactosidase decreases with time Eventually the permease level could fall to the point where some bacteria would have no permease This would be reflected in a decrease in the fraction of the population that is maintained in the induced state when aliquots of such a population are transferred to and grown in medium containing a maintenance concentration of inducer

Such a decrease in the fraction of induced bacteria was observed in the experiment reported in Figure 4 Here a culture pre induced to maximum activity was inoculated into medium containing 2×10^{-6} *M* TMG in one case and 2.5×10^{-6} *M* TMG in the other At various times aliquots were transferred to and grown in medium containing 5×10^{-6} *M* TMG (maintenance concentration) The rate of synthesis becomes fixed at the

saturation value less than maximum, e g at about 6 per cent in Figure 2 These intermediate saturation levels where the fraction of the population in the induced state is constant result from the fact that in the presence of the inducer uninduced bacteria grow more rapidly than induced As a result a steady state level is reached where the number of bacteria becoming newly induced is canceled by an apparent loss of induced cells due to their slower growth The saturation value ultimately reached is given by either

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the steady state $dp/dt = 0$ and the steady state value of p p is given by

$$p = R(p) \qquad (6)$$

Assuming that $R(p)$ is a function of p as in the example illustrated in Figure 5, one sees that there are two possible steady state values for p, i e L_1 and L It is evident however that L_1 is an unstable point since in a bacterium with less than L_1 molecules of permease the level will fall toward zero while in a bacterium with more than L_1 molecules the level will rise toward L

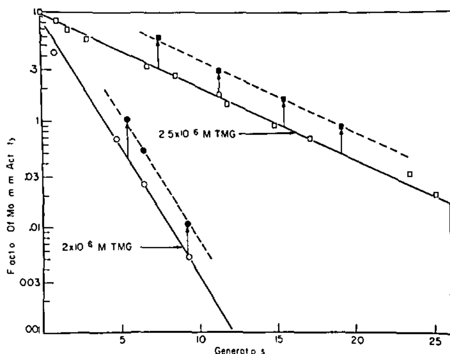


FIG 4 —β Galactosidase content and maintainable fraction (*broken lines*) of a bacterial culture that was pre induced at high concentration of TMG and transferred to indicated low concentrations Fraction maintainable (*s lid poi ts*) was determined by measurement of the enzyme level in an aliquot tran ferred to and cultivated in a maintenance concentration of inducer (5×10^{-6} M TMG)

If permease molecules are randomly distributed among the daughter cells at cell division there will be a certain chance that a cell will get less than L_1 molecules of permease thereby giving rise to a clone of uninduced bacteria Since there is a constant chance in each generation that a cell may thus become uninduced the fraction of the population containing permease should fall exponentially At concentrations of inducer like those shown in Figure 4 L is sufficiently small and near to L_1 that the chance of a daughter cell s getting less than L_1 is appreciable

Working with a medium lacking inducer so as to avoid having to take into

account the production of permease, one can make an estimate of the average number of permease molecules present in a fully induced bacterium For this purpose we again assume that permease molecules are randomly distributed among the progeny at cell division and that a single permease molecule in a bacterium is sufficient for that bacterium to score as maintainable " We also assume that permease is not inactivated during the period in which it is being diluted among the progeny Such assumptions lead to the expectation that after an initial delay the maintainable fraction of the population should fall exponentially at a rate equal to the bacterial growth rate Such an exponential

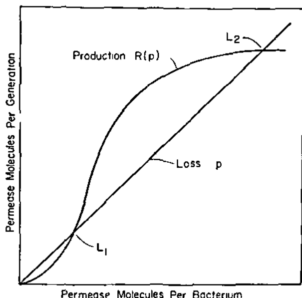


FIG 5 —Hypothetical dependence of rate of production of permease $R(p)$ and rate of dilution as functions of the number of permease molecules in a bacterium

fall should give in a semilog plot a straight line which extrapolates back at zero time (the time of inoculation into the medium with no inducer) to the number of permease molecules per bacterium present initially

Clearly these expectations are not realized in the results of the two experiments shown in Figure 6 one at a generation time of 2 9 hours and one at 10 hours It is seen that the maintainable fraction of the population falls faster than the bacterial growth rate and falls at different rates at the two generation times This behavior would be explained in part if in addition to dilution through cell multiplication destruction of permease occurred perhaps by thermal denaturation Also it is possible that at the low concentration of inducer used for maintenance here, more than one molecule of permease is

necessary to assure the maintenance of a bacterium in the induced state Hence to explain the results in Figure 6 we will assume that under the conditions employed here permease is denatured at a rate such that its concentration would fall to $1/e$ in a time T Furthermore we shall assume that the minimum level of permease which assures induction at the maintenance concentration of inducer is L_1 molecules where L_1 may be greater than 1

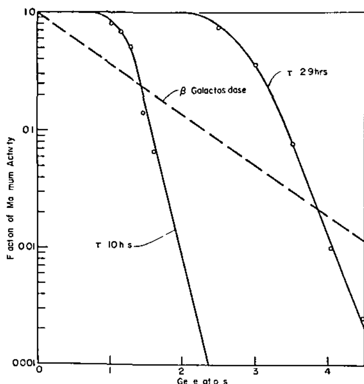


Fig 6 —Maintainable fraction (determined by transfer of aliquots to maintenance concentration of inducer) of a bacterial culture that was pre induced at a high concentration of TMG and transferred to medium containing no TMG Two experiment at the indicated gene ati n times are sho n The β galactosidase level in both ca es fell al ng the indicated broken line

On the basis of these modified assumptions the average permease level p in the bacteria growing in the absence of inducer is given by

$$p = p \ \exp\left(-\frac{t}{\tau}-\frac{t}{T}\right) \tag{7}$$

where p_0 is the number of permease molecules present initially and τ is the generation time $(1/a)$ The fraction Γ_m of the population that is maintainable

(having L_1 or more molecules of permease) can be computed from the formula for the Poisson distribution and is therefore represented by

$$F_m = \sum_{j=L}^{\infty} \frac{p^j}{j!} e^{-p} \tag{8}$$

Substitution in equation (8) of the value p as a function of time from equation (7) gives F_m as a function of time

This expression of F_m as a function of time could be fitted to the results found in the experiment in which the generation time is 2 9 hours The values of T p_0 and L_1 which gave the best fit here were used to calculate a theoretical curve for a generation time of 10 hours This theoretical curve was found to be in fair agreement with the experimental results at the 10-hour generation time The best values of these parameters seems to be $T =$ 3 5 hours $L_1 =$ 2–3 molecules of permease and $p_0 = 300$ molecules It should be remarked that a value of 2 5 hours for T was found in direct ob ervations of the rate at which the capacity of a culture to concentrate inducer was lost under conditions where growth was prevented by incubation in medium lack ing a carbon source as well as inducer (2) In these studies it was observed that no inactivation of β galactosidase occurs even when permease has been re duced by several orders of magnitude Likewise in our observations of the dilu tion of β galactosidase in bacteria growing in the absence of inducer the rate of fall of enzyme never exceeded the bacterial growth rate

It should also be noted that the assumption of a permease threshold of 2 or 3 molecules for a cell to become induced at the maintenance concentration of inducer is not inconsistent with the assumption of a threshold of 1 molecule at the higher concentrations of inducer used in experiments like that shown in Figure 2 It appears that the rate of permease synthesis increases sharply with increase in concentration of inducer (1) and if 2 or 3 molecules give a suf ficiently high rate of permease formation at the maintenance concentration 1 molecule should suffice at appreciably higher concentrations of inducer

Experiments like those in Figure 6 were performed with bacteria pre induced at different concentrations of inducer to see whether the number of permease molecules in an induced bacterium depends on the concentration of inducer The results showed that for all concentrations of inducer equal to or greater than the maintenance concentration the permease level is the same i e 300 molecules per cell

It is possible to examine the compatibility of a maximum level of 300 per mease molecules per cell with the assumption that the critical threshold for induction over a wide range of inducer concentrations is 1 molecule of permease (2 or 3 molecules at the maintenance concentration) If in a bacterium with

300 molecules of permease inducer is concentrated to a level 100 times that in the medium, then in a bacterium with 1 permease molecule the concentration of inducer inside the cell would be 1.3 times that in the medium. An estimate of the expected increase in rate of synthesis of permease resulting from such an increase in internal concentration of inducer can be made in the following way. The rate of formation of permease increases sharply with increase in concentration of inducer. For example, it was observed that the rate of permease formation at a TMG concentration of 10×10^{-6} *M* is 16 times that at 7×10^{-6} *M* (1). Therefore, a bacterium with 1 molecule of permease would be expected to make permease 10–15 times as fast as one with none. Consequently, at concentrations where the chance of getting the first permease molecule is 5 per cent or greater, the presence of 1 molecule leads with high probability to the formation of a second molecule. By this argument the formation of a third molecule will occur with even higher probability, leading to maximum levels of permease. Thus, under these conditions, 1 molecule of permease is sufficient for a cell to become fully induced.

It is evident from these arguments that the value 300 is an upper limit for the number of permease molecules. Were the number larger, then the increase in internal concentration of inducer brought about by 1 molecule would not be sufficient to assure the formation of a second permease molecule at the lower concentrations of inducer.

It can be asked whether the assumed increase in the internal concentration of TMG produced by 1 permease molecule demands too high a turnover number for permease. Estimation of this number requires knowledge of the rate at which TMG leaks out of the cell, since the rate at which inducer is brought in must equal the rate of escape when the internal concentration is constant. This has been reported to be about 7 per cent per minute at 0° (2), and at 37°, assuming that the escape involves only processes of low activation energy, the rate could be 10 times more rapid. Under these conditions a 1.3 fold rise in internal inducer concentration for an outside concentration of 10^{-4} *M* would require a turnover number less than 10,000 times per minute—a not at all unreasonable value.

CONCLUSION

The kinetic phenomena associated with the formation of the inducible enzyme β-galactosidase are in large part explained by the action of galactoside permease, a specific inducible mechanism for the concentration of inducer within the bacteria. Of special interest is the fact that under certain conditions the capacity to form β-galactosidase behaves as an all or none character, individual bacteria in a culture either making this enzyme at maximum rate or not making it at all. Moreover, the descendants of bacteria in the induced

state are also induced while those of uninduced parents are uninduced In this sense the capacity to form β galactosidase and permease behaves like an inheritable characteristic If permease be indeed an enzyme we see an example in which the inheritance of a given attribute depends only on the presence or absence of a specific enzyme

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CHAPTER 7

Mechanism of the Antibody Response*

David W Talmage, M D

ANTIBODY production is a highly specialized form of an individual's adaptation to the environment The mechanism of this adaptive response is unsettled Such diverse substances as the virus of poliomyelitis the pollen of ragweed and arsanilic acid may leave their mark on the pattern of protein synthesized by animals exposed to their stimuli Current thinking on the manner of their influence divides into two viewpoints (1) that the pattern of adaptation is derived from an impression of the environmental stimulus or antigen and (2) that the adaptation is achieved by a selection from pre existing or naturally occurring patterns or antibodies According to the first view the antigen causes the formation of new protein molecules which are different from any molecule which existed in that animal prior to the first introduction of antigen According to the second view the antigen induces an increased production of selected pre existing types of protein molecules In many ways this divergence of opinion regarding individual adaptation is similar to that which divided the Lamarckian and Darwinian schools a century ago in their controversy over adaptation of the species

DIRECT ANTIGEN TEMPLATE THEORY

A widely accepted form of the first view considers the antigen as the direct governing force in the folding of the antibody molecule and for this reason might be called the direct antigen template theory of antibody production (1–8) The postulation of a direct impression of the antigen on the configuration of the antibody requires the persistence of at least a few molecules of antigen as long as antibody production continues Since it is impossible to prove the absence of a few molecules of antigen it is impossible to disprove directly the direct antigen template theory It must be said with equal candor that no conclusive evidence exists for an alternative theory of antibody production For

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this reason most workers in the field prefer to keep the direct template theory because it serves them as a useful model In the discussion that follows deliberately slanted views are taken first in favor of the indirect antigen template theory and then in favor of the natural selection theory It is hoped thereby to stimulate discussion and to clarify the issues

INDIRECT ANTIGEN TEMPLATE THEORY

Burnet and Fenner (9*a*) have proposed that exposure to antigen so modifies the enzymes responsible for globulin synthesis that these enzymes produce a new globulin complementary to the antigen that induced the change This might be considered an indirect antigen template theory since the pattern or specificity of the antibody is obtained from the antigen indirectly through some intrinsic synthesizing system Basically, however it is similar to the previous theory in holding that the pattern of response is obtained from an impression of the environmental stimulus Schweet and Owen (10) have recently proposed that the antigen produces a permanent hereditable change in the desoxyribose nucleic acids (DNA) of the nucleus of the antibody producing cell The specific information of the new DNA is then passed on to the RNA of the cytoplasm where in the presence of more antigen the RNA is induced to synthesize antibody In a recent book (9*b*) Burnet makes somewhat similar changes in his original theory With these modifications the indirect antigen template theory is brought partially into line with recently obtained knowledge concerning the mechanism of induced enzyme synthesis In addition to this analogy to a known biological system a number of reasons exist for favoring the indirect over the direct antigen template theory These all point to antibody synthesis by a replicating unit which is an intrinsic part of the cellular apparatus

1 The number of antibody molecules produced in 48 hours may be as many as 100 000 times the maximum number of antigen molecules which during that time could be left in the entire body (11) This approaches one molecule of antibody per antigen molecule per second which approximates the rate that Haurowitz estimated for antitoxin production (12) It is difficult to conceive of a mechanism by which the direct antigen template could accomplish this degree of amplification of response to stimulus Even discounting the evidence that protein synthesis is slow relative to other enzymatic processes (13) and assuming that one antibody molecule can be formed each second on each antigen template two problems remain (*a*) that of concentrating the antigen at the site within the cell where antibody is being formed and (*b*) that of preventing the formed antibody from blocking the template A solution to the first problem requires a pre existing antibody like receptor and the solution to the second problem requires that even the antibody be unable to bind the antigen

2 A second injection of antigen may give a faster response and up to 100

times the amount of antibody as the first injection (14, 15) One might postulate that a more favorable distribution of antigen is responsible for the anamnestic response were it not for the fact that the anamnestic response can be induced by an amount of antigen which can be completely neutralized by the circulating antibody remaining from the primary response (11 16)

3 In a heterogenic group of animals (rabbits) the distribution of antibody titers to the same dose of antigen is normal when plotted on a logarithmic scale (17 18) but highly skewed when plotted on an arithmetic scale (17) This suggests that the number of replications of the template not the number of templates is normally distributed

4 Following a second injection of antigen there is an exponential rise in circulating antibody (9*a*) The work of Taliaferro (19) indicates that changes in serum antibody concentration suitably corrected for metabolic decay truly reflect the rate of antibody synthesis

NATURAL SELECTION THEORY

If antibodies are synthesized by replicating cellular units there is considerable advantage in postulating that a few units for each synthesizable molecular type existed prior to the first injection of antigen Such a theory would permit a closer analogy to bacterial enzyme synthesis which is now thought to involve the induction of inherited enzyme synthesizing units (10 20–22)

A close biological parallel to adaptation of the individual is adaptation of the species Since the latter is now generally considered to be due to a selection of its individual but different members it would not be surprising if adaptation of the individual were based on a selection of its individual diverse components —cells and molecules When muscles are used excessively they hypertrophy There is nothing to suggest that this type of adaptation is anything more than the overactivity of a pre existing synthetic process Even overactivity of a pre existing function cannot be passed on to progeny except as a temporary state in unicellular organisms (23) To the limit of present knowledge adaptation of individuals and species always occurs as a selection of naturally occurring processes

There is a good reason for the dominance of natural selection in the various types of adaptation A living organism is so complicated that any change in its makeup has a possibility of being harmful It is for this reason that increased rates of mutation are feared (e g from ionizing radiation) Similarly any hereditary process which could be easily changed by the environment is likely to be self destructive On the level of the individual the production of an entirely new cell or a new molecule has some probability of being self destructive A new cell may be a host destroying cancer and a cell which can make new molecules at the demand of the environment will sooner or later destroy itself

unless some control exists to prevent the formation of autotoxins or 'auto antibodies' In addition to the rarity of antibodies against autoantigens evi dence that a self protective mechanism exists is found in the fact that under certain circumstances the injection of antigen may lead to persisting and specific unresponsiveness to that antigen These conditions are (1) during the fetal or newborn period (24), (2) after X radiation (25) and (3) the ingestion of haptenes (26) This phenomenon of self protection requires an ability to dis tinguish between self and foreign which in turn requires a complete set of receptors to recognize either one s own molecules or else every conceivable foreign configuration While the recognition of self as suggested by Burnet (9a) may seem at first glance the simpler of the two alternatives in actual operation it may present an impossible task For one thing the recognition of self would require a blocking at the site of antibody production of every part of homologous molecules i e the molecule could not be rejected or accepted as a whole on the basis of a single marker representing a small fraction of the molecule s surface For example the injection of a haptene attached to homol ogous protein results in antibody to the haptene only and not to the homol ogous protein (27) In addition the number of different receptors would have to be very large to recognize the tremendous diversity of homologous molecules Every site of antibody production would have to carry a complete set of these receptors and a mechanism to prevent overloading by the tremendous excess of certain antigens such as albumin To account for induced unresponsiveness new receptors would have to be produced during times of reduced adaptability

The alternative to this self marker system is a method of recognizing foreign substances This recognition could be accomplished by a set of re ceptors capable of binding almost any conceivable foreign substance together with a mechanism which selectively multiplies these receptors on demand and excludes or inhibits self binding receptors The natural selection theory postu lates a wide diversity of naturally occurring antibodies and consequently does not require a self marker system The foreign marker' system of the natural selection theory has the advantage that recognition of all substances would not be required of every antibody producing cell In addition tolerance and hypersensitivity may be considered as opposite aspects of the same process the one being due to an inhibition and the other to a stimulation of replication

It is not surprising that attempts have been made to consider antibody pro duction as a process of natural selection Although the term ' natural selection to describe antibody production was first used in 1955 by Jerne (28) it was more than fifty years ago that Ehrlich (29) advanced his side chain theory of antibody production Figure 1 is taken from Ehrlich s address to the Royal Society in 1900 and illustrates clearly his concept of the natural selection

process The antigen through a chance affinity binds certain receptors or side chains on the surface of a cell The cell replaces the one bound side chain with two new ones and the process continues until there is a great excess of side chains which are cast off into the circulation as antibodies This theory received a serious setback when it was shown experimentally that antibodies could be formed against a great variety of synthetic substances (27) A seemingly irref

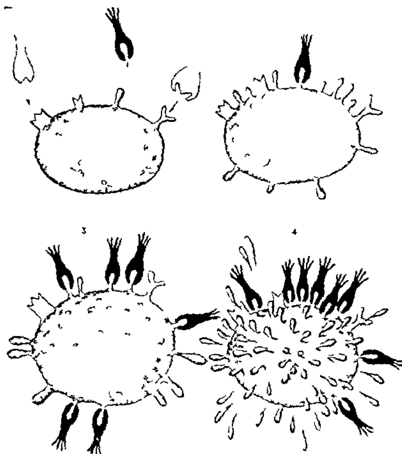


Fig 1 —Ehrlich's conception of the multiplication and shedding of natural cellular receptors after their combination with antigen (29)

utable argument was advanced that the cell could not possess a different side chain for each conceivable chemical that might be synthesized Increasing knowledge of genetics and intermolecular reactions however now permits an answer to this argument by demonstrating that it overestimates the number of different antibodies required and underestimates the capabilities of the heredi tary process

One defect in Ehrlich s ideas may have been in his concept that each pre existing antibody was made for a specific purpose A pre-existing antibody to a synthetic chemical had no conceivable purpose and when such an antibody was found his whole theory was discarded In the struggle for existence how ever animals survive not through foreknowledge but through chance ability to cope with the unforeseen dangers lurking ahead In a constantly changing environment there should be a selection of those with the greatest variety of preadaptabilities In this sense an antibody to arsanilic acid has as much pur pose as an antibody to an undeveloped strain of virus

Ehrlich also made the mistake of trying to combine the concepts of natural selection and absolute specificity Because of the almost unlimited number of antibody responses which the same animal can make this is obviously impos sible However instead of discarding the concept of natural selection it may be possible to eliminate the concept of absolute specificity It has been shown that a single antigen can combine with a whole spectrum of antibodies (27 30) and conversely that a single antibody can combine with a whole spectrum of antigens Instead of the unlimited number of different reactive sites which was presumed to exist when the antigen template theory was first formulated Haurowitz (31) has recently estimated that the total number of different reac tive sites on antibodies may be less than 50 000 In view of the large amount of information contained in the chromosomes it is no longer possible to dismi s the idea that a large variety of globulin molecules containing a substantial frac tion of the possible antibody sites is synthesized in the absence of any informa tion provided by environmental antigens The number of different antibody sites may be much larger than the number of different antibody molecules since the two similar valence sites of most antibodies occupy only a small fraction of the total surface of the molecule

The supposition can be made that when an antigen containing a synthetic chemical group is exposed to a large heterogeneous population of reactive sites *on globulin molecules some of these sites through chance alone will have an* affinity for the new configuration The degree of affinity for antigen of the randomly selected reactive sites should vary with a normal distribution If the number of different reactive sites is large enough there should be a few which have a high degree of affinity for any conceivable chemical grouping Any mechanism of selectively multiplying those protein molecules with a high af finity for antigen will result in the production of antibodies

This concept of antibodies is substantiated by the finding of a wide spectrum of affinities with which different antibodies bind the same antigen (28 32) Consequently it is necessary to consider the specificity of antibodies as relative and determined by the degree of affinity between antigen and antibody This type of specific affinity between naturally occurring substances is common in

nature The action of enzymes is based in part on an affinity for the substrate Toxins and viruses may have a highly specific affinity for cells with which they have had no previous contact Penicillin (33) and isoniazid (34) have affinity for bacteria sensitive to their action The uniqueness of antibodies lies not in their relatively specific affinity for antigens but in the manner in which their con centration can be increased in response to an environmental stimulus

To summarize the natural selection theory of antibody formation has the following inherent advantages over the indirect template theories of Burnet and Fenner and Schweet and Owen (1) it represents a closer analogy to induced enzyme synthesis in bacteria and to the general processes of adaptation of indi viduals and species (2) it obviates the postulation of a specifically induced mutation of somatic cells (3) it obviates the postulation of a self marker di rectory in every antibody producing cell, and (4) it gives meaning to the ob served diversity of serum globulins

If the natural selection theory has these inherent advantages and if the hereditary apparatus is sufficiently diverse to explain antibody production on this basis it is necessary to consider the direct experimental evidence for this position and a possible model of the process Unfortunately the theory has not been generally considered as one of the possible theories of antibody production and very little experimental work to test its validity has been done It is pos sible however to point out two approaches to the problem which give promise of rewarding results

The first of these lies in the relation between heredity and antibody produc tion It is well known that a good antigen for one species may be a poor antigen for another It is also generally considered that homozygous animal strains give a more reproducible antibody response than do heterozygous strains A few definitive studies have been done on developing strains of laboratory animals which are selected for their ability or inability to produce antibodies to a single antigen (17 35–37) It would be useful to know whether the ability to produce antibodies when two such strains are crossed is genetically determined How ever it may be difficult to study a single antibody in this kind of experiment since antisera contain a spectrum of antibodies An even more difficult problem may be that of distinguishing the inherited inability to produce antibodies to an antigen from tolerance to that antigen induced by cross reacting auto antigens

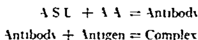
A second and perhaps more definitive test of the natural selection theory would be the demonstration of antibodies to synthetic chemical haptens in the serum of unimmunized animals Since these antibodies if present at all are obviously at a very low concentration it is necessary to use techniques which are unusually sensitive It is interesting that extremely sensitive techniques such as agglutination hemolysis or Jerne s bacteriophage stabilization test (38)

have demonstrated antibodies in normal serum Because of the importance imputed to antibodies to synthetic chemical it would be desirable to demonstrate antibodies to these substances It is possible that techniques using radioisotopes may provide the answer

THE MECHANISM OF THE ANTIGENIC STIMULUS

The preceding paragraphs have dealt largely with the origin of the specificity of the reactive site on the antibody molecule According to the indirect antigen template theory this is achieved by a change or mutation of existing globulin synthesizing units Once this change has been effected there is no essential difference between this theory and the natural selection theory Antigen is needed in both instances to induce the replication and synthesizing activity of these units For this reason it seems desirable to separate the problem of the role of antigens in this latter process from the three general theories of antibody production already discussed It is not necessary to accept Ehrlich s picture of attached cellular receptors or Jerne s concept of replication of circulating globulins in order to accept the theory of natural selection (39) Conversely a proof of one particular mechanism by which antigen stimulates replication of the antibody synthesizing units will not decide the issue between induced mutation and natural selection

The most obvious reaction that takes place following the injection of antigen into a previously sensitized animal is the binding of the antibody by the antigen The result of the reaction between antigen and antibody is the rapid metabolism of both antigen (40) and antibody (41) and a sudden reduction in the concentration of antibody For this reason it might be conceived that antibody is the specific inhibitor of its own production (a common chemical and biological phenomenon) and that the role of antigen is the binding of an inhibitor For this reaction we may write


A S U + A A = Antibody
Antibody + Antigen = Complex

A hypothetical antibody synthesizing unit (A S U) plus amino acid (A A) leads to the formation of antibody molecules This reaction becomes inhibited when the antibody concentration is built up sufficiently but it continues when antigen combines with and removes the antibody It is obvious however that this model alone is too simple to explain the experimental evidence for a replicating unit Some feedback is needed to stimulate the replication and control the concentration of A S U Similar feedbacks are used as homeostatic mechanisms to control the level of most humoral substances For example the rate of adrenal steroid production is controlled by the inhibition of the steroid of

adrenocorticotrophic hormone Szilard (42) has suggested that a mechanism similar to that proposed by Vogel (43) for enzyme suppression may serve both as a feedback and as a means of amplifying the response to a minimal stimulus According to this model antibody production by nucleic acid templates and *the replication of these templates is inhibited by the product of an enzyme* nearly identical in configuration with the antibody The antigen by combining with the enzyme prevents the production of repressor This model would provide a means of control and explain the lag after exposure to antigen which precedes the production of antibody and also the lag after elimination of the antigen which precedes the sudden decline in the rate of antibody production

Whatever the exact mechanism of induction experimental evidence does exist which *indicates* that it is the binding of pre existing antibody or something similar to it that induces the antibody response Perhaps the most direct and best supported evidence in this regard is the change in character of the antibody formed during the course of immunization in an animal There is considerable unanimity of opinion that the later antisera contain greatly increased amounts of a more avid or more tightly binding antibody Although there is reason to think that both early and late sera contain a mixture or spectrum of relatively avid and non avid antibodies the change in composition of the sera toward the more tightly binding antibodies indicates that production is in some way *related to binding* It would appear that the greater the binding to antigen the greater the stimulus to production Using techniques developed by Farr (44) it has been possible to measure the dissociation constants between antigen and the predominant antibody type in primary and secondary response sera A plot of the fraction of antibody bound describes a steep curve with changes in the concentration of free antigen so that for each antibody type there is a fairly narrow critical range of antigen concentration in which the fraction of antibody bound to antigen changes from low to high Since in the region of antigen excess this *curve is independent of the amount of antibody present it is possible to calcu* late the fraction of antibody bound even when the antibody itself cannot be measured Using bovine albumin as an antigen in rabbits it has been found that there is a critical dose of antigen below which the response of a given type of antibody falls off very sharply Because of the spectrum of antibodies in any serum and the uneven distribution of antigen in the body it is not possible to determine with precision the relation of in vivo antigen concentration to the response of any particular antibody type Two points however can be made (1) that the critical dose of antigen establishes an initial serum concentration of antigen which would bind a high percentage of any antibody that might be present and (2) that the critical doses for the primary and secondary responses have approximately the same ratio as the dissociation constants or binding curves of the antibodies produced

In the case of the secondary response, the existence prior to the injection of antigen of a measurable amount of antibody permits a determination of the relationship between antibody binding and antibody response In this case it *has been possible to achieve a tenfold increase in antibody level with an amount* of intravenously injected antigen which lowers the circulating antibody only 50 per cent Because the antigen antibody aggregates are probably concentrated a higher fraction of antibody might have been bound in certain tissues or cells Two additional observations should be noted (1) the primary response to an intravenous injection of an insoluble antigen can be reduced considerably by maintaining the antibody concentration with passively administered antibody (45) and (2) the anamnestic response cannot be initiated regularly without the use of antigen either by lowering the concentration of circulating antibody with exchange transfusions or by transferring cells from a sensitized animal to the low antibody environment of an untreated animal The failure of induction in the latter instance indicates that the suppressing effect of antibody in the first instance is the result of binding of antigen and not due to a direct effect on the intracellular synthesis of antibody

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CHAPTER 8

On the Synthesis of Antibody Protein in Vitro*

Herbert S. Anker

It is generally agreed that antibody formation is associated with organs containing relatively large numbers of lymphoid cells (1). Among these organs, the spleen in particular participates in antibody formation especially when antigens are administered intravenously. Formation of antibody also occurs in non-immunized, immature, or X-irradiated recipient animals into which spleen cells from immunized donors are injected. Previous observations (2) indicate that in the rabbit the major proportion of antibody protein in an anamnestic response is synthesized between the third and fifth days following intravenous injection of a soluble antigen.

In vitro observations with spleen explants or slices have been restricted thus far chiefly to hemolysins (3, 4), typhoid (5) and paratyphoid (6) agglutinins and diphtheria antitoxins (7).

The method reported here permits the synthesis of antibody proteins by rabbit spleen cells on incubation for several days in vitro. It was found that incubation of surviving tissues by shaking in a suitable gas phase is unsatisfactory for prolonged survival of spleen cell suspensions, apparently because of mechanical damage to the cells by the agitation. Since antibody was not found it appears that intact cells are required for the synthesis.

To avoid damage by agitation, spleen cells were incubated at rest. To overcome the limitations of gas diffusion in a large volume of medium, the cells were supported in a thin layer by a semipermeable membrane. The layer of fluid above the cells was only about 1 mm thick. The large volume of medium in contact with the underside of the membrane served as a reservoir of nutrient materials and permitted the removal of metabolic products. Exchange of ma-

* Supported by a grant from the United States Atomic Energy Commission.

terials was enhanced by stirring On incubation of spleen cell suspensions by this method cell degeneration was minimized and antibody was formed in such amounts that it could be precipitated directly Since the antibody protein could not diffuse through the membrane into the reservoir it was found in all experiments only in the medium in which the cells were suspended

Antibody formation was considerably lower if the spleen was removed 2 days after a secondary antigen injection When the spleen was removed after 24 hours no antibody formation could be detected although all the cells were histologically fairly well preserved for 4 days The formation of polyblasts and multinucleated giant cells after 5 days of incubation in vitro indicates that for this period of time the medium presently used does not sustain the cells in the same histologic state as is maintained in the intact organ in vivo The inadequacy of the culture medium probably accounts in part for lack of antibody formation when the spleen is removed prior to the second day after antigen administration Similar effects had been reported previously (3) and attributed to a deficiency in the culture system

Even though serum is not absolutely essential for the synthesis of antibody protein extracellular protein appears to contribute to the preservation of the cells It has likewise been noted that serum is necessary for maintenance of growth in many cell cultures (8) It cannot be decided at the present time whether the serum effect is due to the protein itself or to low molecular weight factors associated with it

Taliaferro and Talmage (9) have studied the anamnestic response of rabbit spleen cells removed 3 days following a secondary challenge with BSA after transfer to non immunized recipients In these experiments a total of from 4 5 to 50 mg of antibody protein per spleen was synthesized following transfer of one spleen to one or two animals after a challenging dose of 60–90 mg of antigen or about four to five times the amounts used here Although the magnitude of the antibody response as a function of the antigenic stimulus is not known the amount of antibody protein synthesized in vitro in the experiments reported here ranged from 3 to 37 mg per spleen The yield in previous in vitro experiments (7) was only about one tenth the amount of antibody obtained in equivalent transfer experiments in vivo

In all previous in vitro experiments (3–7) antibody formation could be demonstrated only because of the high potency of the antigens used and because of the very sensitive immunologic methods available for the detection of antibodies against particulate antigens and toxins

The method of incubation described here would seem to hold promise for other experiments requiring the maintenance of differentiated cells in a functional state in vitro

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CHAPTER 9

A Hypothesis concerning the Roles of Particulate and Soluble Ribonucleic Acids in Protein Synthesis*

Mahlon B Hoagland,† Paul C Zamecnik and Mary Louise Stephenson

In 1939 when it was generally thought that a reversal of proteolytic reactions might be the key to protein synthesis Linderstrom Lang wrote The mechanism of the synthesis of protein in the living cell is very obscure and can only be clarified in connection with the study of coupled reactions in the metabolic system (1) Two years later the now classic paper of Lipmann (2) suggested that peptide bond synthesis might be effected by coupling with energy yielding reactions involving phosphate bonds Since then a growing body of evidence has provided strong support for the view that the labile phosphates of ATP[1] are used in the synthesis of peptide linkages (3–8) and of true alpha peptide bonds (8–11)

Parallel developments over a similar span of years pioneered by Caspersson (12) and Brachet (13) have brought to light much circumstantial evidence implicating cellular ribonucleic acid in protein synthesis In animal tissues ribonucleic acid exists largely associated with protein in discrete fragments operationally defined as microsomes which are apparently derived from the endoplasmic reticulum or ergastoplasm described by the electron microscopists (14–19) That these microsomes are the initial site of protein synthesis in the

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1 The following abbreviations are used throughout this paper RNA ribonucleic acid ATP and GTP the triphosphates of adenosine and guanosine PEP phosphoenolpyruvate AMP adenosine 5 phosphate

mammalian cell seems highly probable from the results of in vivo studies on incorporation of C^{14} labeled amino acids into the protein of centrifugally separated cellular fractions (20–24) Furthermore, experiments in this laboratory (25 26) and in England (27) have shown that the RNA rich ribonucleoprotein particles of the microsomes which are obtained by removal of the lipid portion of the microsomes with deoxycholate or high salt concentrations incorporate amino acids into their protein moiety considerably more rapidly than do the whole microsomes (For recent comprehensive reviews of this field including valuable information of a similar kind from work with micro organisms see [13] and [28])

More direct evidence for the involvement of RNA in protein synthesis has recently been reported by us (29 30) A review of the more pertinent aspects

TABLE 1

REQUIREMENTS FOR FORMATION OF LABELED PROTEIN IN *pH 5* FRACTION-MICROSOME SYSTEM (KELLER AND ZAMECNIK [31])

	C 1 pe M pe Mg P ot	
Complete system	167	171
Complete system minus microsomes†		2
Complete system minus pH 5 fraction†		16
Complete system minus pyruvate kinase		10
Complete system minus PEP		13
Complete system minus ATP		2
Complete system minus GDP		25

Th compl t sy t n c t d m rosom pH 5 fra u 0 03 mg f p u t k 0 1 μm l of L l C (82 000 p m) 10 μm les f PEP 0 5 μm l of ATP d 0 25 μm l f GDP fi l lum of 1 0 ml ; cul tio 10 m tes t 37 Th final l m f ll th flasks was bro ght t 1 0 ml by th dd t *10 15 M KCl*

† Add d ft th c b t t g th m m t f protei 1 5 mg all th fla ks f th C assay

of this work will be facilitated by reference to Table 1 (31) This defines the requirements of the cell free system of rat liver in which C^{14} labeled amino acids are incorporated irreversibly into peptide linkage in protein Most of the radio active amino acid incorporated into this system, as indicated above is found in the protein of the microsome fraction Soluble enzymes which may be concentrated by precipitation at pH 5 2 (the pH 5 fraction ') are required in the system and are known to activate amino acids by use of the AMP pyrophosphoryl bond of ATP as shown in Figure 1 (cf 32–35) It has been concluded that, following amino acid activation the bound intermediate was transferred to microsomes where peptide condensation occurred (32, 33) There is an as yet unexplained requirement for GTP in this latter reaction Using this system, we noted (30, 31) that ribonucleic acid *also* became labeled additively

with amino acids We were very much surprised however to find that the RNA so labeled was predominantly *not* microsomal, which made up some 90 per cent of the RNA present in the reaction mixture but was RNA associated with the pH 5 fraction (referred to as soluble RNA or SRNA) (see Table 2)

We re examined the literature to find out what was known about soluble non sedimentable cellular RNA We discovered that it had been studied and characterized by several workers (for a review see Smellie [36]) It appears to be distinct from both nuclear RNA and microsomal RNA in its metabolic turnover

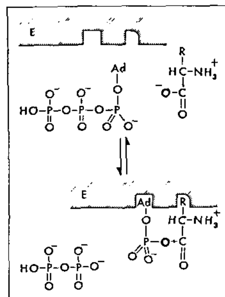


FIG 1—Schematic representation of amino acid carboxyl activation by ATP and the pH 5 fraction Ad = adenosine The heavily drawn O indicate the attacking carboxyl oxygen which would remain with the nucleotide moiety upon subsequent splitting of the activated compound (*dashed line*) From Hoagland Keller and Zamecnik (32)

of P^{32} and nucleotide base precursors It represents some 20 per cent of total cellular RNA

There has now accumulated a considerable body of evidence on the nature of this soluble RNA and of its reactions with amino acids This is briefly summarized herewith and for details the reader is referred particularly to recent reviews (37 38 28) and original papers (39–43)

Estimates of the size of SRNA indicate a molecular weight of 10 000–40 000 and a heterogeneity of composition Amino acids following their activation by the mechanism depicted in Figure 1 are transferred reversibly to a covalent linkage on SRNA by their specific activating enzymes Each amino acid appears to be bound to a specific site as there is no competition between L amino acids

for binding sites It is likely that amino acids are bound each to a different SNRA molecule The amino acid is found linked to the terminal nucleoside residue of the SRNA by esterification of its carboxyl group on the 2′ or 3′ hydroxyl group of ribose (44–46) It has been shown that this terminal nucleotide is adenosine and that it is linked in turn to a cytidylic acid in the usual 3′,5 phosphodiester bond The immediate precursors of these end groups are the triphosphates of adenosine and cytosine This same terminal configuration of nucleotides seems to be necessary for attachment of all the amino acids, which means that the specificity of amino acid binding must depend on a base sequence internal to this grouping Specific soluble enzymes other than amino acid-activating enzymes are responsible for the reversible attachment of these terminal groups to SRNA It is important to observe that microsomal RNA,

TABLE 2

LABELING OF RNA WITH C^{14} LEUCINE IN THE MICROSOME pH 5 FRACTION SYSTEM

	System	Counts per Min per Mg RNA
1	pH 5 fraction plus microsomes	1 220
2	pH 5 fraction plus microsomes ATP omitted	311
3	pH 5 fraction	2 130
4	Microsomes	168

* The [illegible] mpl t [illegible] b t [illegible] mi t [illegible] ta ed mi osomes pH 5 f a t [illegible] phosph e lpyru t pyru t k nase ATP d C [illegible] a l [illegible] bo t b ff [illegible] nta g mag es m [illegible] l cul t wa f r 10 mi utes at 37 Aft r th [illegible] ul t [illegible] m rosomes w added t fla k 3 a d pH 5 fract [illegible] t fla k 4 RNA w [illegible] xtracted w th NaCl f fte [illegible] (30))

either intact or partially degraded does not have a similar capacity to bind amino acids or terminal nucleotides

In vivo work in mammalian (41) and bacterial (47, 48) systems has shown that amino acids become linked to SRNA prior to their appearance in any other isolated cellular RNA or protein fraction In bacteria under conditions of inhibition of protein synthesis (by chloramphenicol or deprivation of an essential amino acid) amino acids accumulate on SRNA Table 3 shows furthermore that in vitro once the SRNA has obtained its C^{14} amino acid it is capable of transferring it to microsomal protein in the presence of GTP, ATP and soluble enzymes Not only is the amino acid found to appear in microsomal protein, but, concomitantly at least some of the SRNA becomes incorporated into the microsomal particles as shown by experiments with isolated SRNA which had been biosynthetically labeled in its pyrimidine bases using C^{14} orotic acid Again, GTP is required for this reaction

How may we construct a hypothetical sequence of reactions which will account for these observations and at the same time furnish a framework on

which to account for the ultimate sovereignty of the genetic material over protein structure?

One unpleasant possibility is that SRNA is a metabolic reservoir of activated amino acids not on the direct pathway of protein synthesis and that microsomal RNA is the true recipient of amino acids Our failure to observe a labeling of microsomal RNA would be indicative of its metabolic competence i e, any amino acids so obtained would be immediately transferred to protein If such is the case how is SRNA able to transfer its bound amino acids to microsomal protein? Since some residual activating enzymes are known to be contaminants

TABLE 3

TRANSFER OF C^{14} LEUCINE FROM PRELABELED ACTIVATING ENZYME FRACTION TO MICROSOMAL PROTEIN

	TOTAL COUNTS	
	RNA	Protein
Complete system (before incubation)	489	30
Complete system (after incubation)	180	374
Complete system *minus* GTP	111	40
Complete system *minus* generating system	72	155
Complete system *minus* both GTP and generating system	23	30
Complete system *minus* generating system but with 5×GTP	145	179
Complete system CTP replacing GTP	96	44
Complete system UTP replacing GTP	101	53
Complete system *plus* 0 005 *M* C leucine	183	314

Th mpl t yst m t ed p l b led pH 5 zym f t m m ph ph lpy v t pyru t k as d GTP Th pH 5 zym f ac t w p l b l d by t t g 10 m tes w th ATP d C l f ll d b tw p ec p tat f th m f m d l ted act m t e t pH 5 2 (1 m lf gl l 7 m k d St ph [29])

of even the most carefully prepared microsomes it is conceivable that SRNA–amino acid might by reversal of the reaction which formed it produce amino acyl adenylate which would then transfer amino acid to protein via intact microsomal RNA This possibility must be left open but would appear to be unlikely on the grounds (1) that the binding sites on SRNA for amino acids are highly specific and (2) that a part of SRNA appears to accompany the amino acids into the microsomes

Let us turn to a more intriguing suggestion that SRNA is a true intermediate in protein synthesis (Fig 2)

We make the general assumption that RNA is the template for protein synthesis i e that the final linear disposition of amino acids with respect to one another in a peptide chain will have some direct relationship to the sequence of bases in RNA (The reader is referred particularly to Gamow 49 and Crick

et al 50 for theoretical discussions of the coding problem) In our figure the SRNA molecule is depicted as having a certain specificity (symbolized by its unique contour) which is determined by a sequence of bases The terminal piece common to all the SRNAs shown represents the AMP CMP end The amino acid–activating enzymes would be expected to bear three specific reactive sites—one for the adenine of ATP one for the R group of the amino acid it activates and one which permits recognition of its specific SRNA That the specific interaction of SRNA and activating enzyme is a function directly

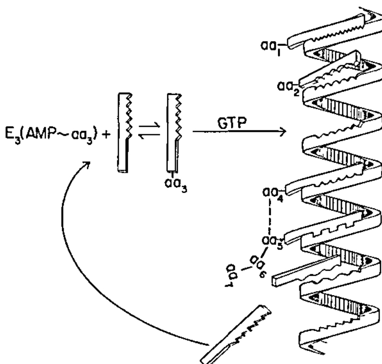


FIG 2—A scheme for the interaction of microsomal RNA and soluble RNA–amino acid

or indirectly of the base sequence of SRNA seems highly probable but of course remains to be proved Thus the sequence *AGU* for example reacts only with activating enzyme 1 *GAC* with enzyme 2 and so on up to 20

Following the formation of the specific amino acyl adenylate–enzyme intermediate conditions are established for binding of SRNA to the enzyme This results in the transfer of amino acid from acyl phosphate linkage to ester linkage on the 2 or 3 hydroxyl group of the terminal adenosine of the SRNA The transfer is accompanied by the release of the AMP moiety of ATP

These SRNA–amino acid compounds exist in the milieu bathing the microsomal particles In order for them to find the proper locus in the particles in a

time short enough to account for the speed of protein synthesis they should be present in high concentration as should the microsomal particles themselves Although there may well be other unknown factors favoring the likelihood of collision between SRNA–amino acid and particles it is of some interest that both activating enzymes and amino acid–binding RNA make up a large fraction of the total non particulate cellular protein and RNA Furthermore under conditions of rapid growth there is generally an increase in the number of ribonucleoprotein particles per cell These factors may contribute to bringing the activated intermediate to the proper site in the particle

The basic concept upon which this hypothesis is based is that *a certain polynucleotide fraction of SRNA with its bound amino acid locates itself by hydrogen bonding on a limited segment of the high polymer RNA of the ribonucleoprotein particle to which its base sequence is complementary* This is represented in Figure 2 by the complementary contours of the reactive segment of SRNA and microsomal RNA This interaction would be highly specific and would be analogous to the base pairing in the double stranded DNA molecule GTP must function at this point in the sequence in an as yet unknown way An implication of this hypothesis is that microsomal RNA might be at least partly single stranded

A problem arises in regard to the size and conformation of our carrier polynucleotide On theoretical grounds a trinucleotide or tetranucleotide might suffice to code for an amino acid We find experimentally however that SRNA is a relatively large molecule of perhaps 30 nucleotides in length Furthermore we find that the amino acid is not attached directly to a specific coding sequence but is linked to the end of a pair (or possibly a triplet) of nucleotides which is common to all the RNA carriers At the moment the only way to circumvent the large size of the SRNA is to suggest that all of it does not enter the microsomes and that the active fragment is split off prior to its admission to the template An answer to this question may be obtained by a study of the exact amount of SRNA which enters microsomes with a known quantity of amino acid Preliminary experiments of this kind do suggest such a limited transfer The common terminal configuration of nucleotides might serve as a reaching device when the adapters are on the template permitting amino acids to cover the gap necessary to permit peptide condensation

Judging from the probable size of the coding units of SRNA we are dealing with it seems that the amino acid residues would be spaced too far apart to permit linear condensation *along* the RNA backbone Thus we should have to assume a structure of microsomal RNA which would bring the amino acids close enough together to condense—perhaps a flattened helix on which amino acids would line up parallel to the screw axis as suggested in the figure It should be emphasized that this suggestion is only symbolic of the idea that discontinuous units of SRNA react specifically with the longer microsomal RNA

polymer We must learn much more about the arrangement of RNA in the microsomal particle before a definitive scheme may be drawn As a final step the SRNA molecule would return to the soluble milieu to recycle and the polypeptide chain would spontaneously fold into its characteristic secondary and tertiary structure

The scheme embodies the concept that SRNA is structurally related to microsomal RNA each molecule of SRNA (or of its functional part) is a structural complement of a segment of the microsomal polyribonucleotide The latter RNA must be a relatively stable master pattern containing the full sequence of bases required to code for the proteins it synthesizes This information must in turn be derived from the nuclear DNA How is the information in the microsomal RNA template conveyed to the SRNA so that it knows which sequence of bases it must have in order to attract a particular amino acid? One suggestion is that particle RNA may be synthesized on a DNA template as a double stranded structure which is then transported to the extranuclear environment Alternatively it might be synthesized by DNA as a structure which could replicate a complement of itself In either event in association with protein in particles half the double strand may be enzymatically broken down (perhaps by a ribonuclease like enzyme) into soluble subunits (SRNA) which go forth to react with amino acids after having had their terminal group of nucleotides attached by soluble enzymes Since these fragments originated as structural complements of segments of microsomal RNA they would have the configuration necessary to permit them to recognize their specific locus when they return to the particle with their attached amino acid Thus genetic continuity would be maintained from the nucleus via the microsomes to events occurring in the soluble phase

At the time that this kind of hypothesis was originally presented (March 1957) the authors favored the idea that an SRNA molecule might bind more than one amino acid perhaps on internucleotide phosphate or hydroxyl group The SRNA would thus have a role as a primary template as it would arrange a limited number of amino acids in sequence Different SRNA s would thus have different sequences of amino acids This reaction would then be followed by the complementary base pairing with microsomal RNA (the secondary template) described above The primary template role of SRNA seems however to be unlikely on the basis of the more recent findings indicating that the amino acid links to the terminal nucleotide of a specific RNA

Before we had suggested a hypothesis to explain our findings—in fact, before the discovery of the relation between SRNA and amino acids—Crick (37) had come to a conclusion identical with the present proposal on theoretical grounds He argued that if the high polymer RNA of the microsomes were in

deed a template for protein synthesis it would be necessary to postulate an initial reaction between amino acid and an adapter molecule which would be more readily recognized by the template It was felt that the R group of an amino acid alone would not have sufficient individual identity to be located on the template with the precision intrinsic in the protein synthetic process A small polynucleotide of base sequence complementary to a segment of the template would ideally serve as such an adapter for it has the necessary groups to react with amino acids and also to react by highly specific hydrogen bonding with a complementary base sequence on the template

We are pleased to thank Drs Jesse Scott Liselotte Hecht Elizabeth Keller Robert Loftfield James Watson Francis Crick and Sydney Brenner for valuable discussions

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CHAPTER 10

Aspects of Ribonucleic Acid Synthesis

Martynas Yčas

There is no Majesty and there is no Might save in Him the Glorious the Great We shall assuredly perish miserably and none will know of us — *Third Voyage of Sindbad*

SINCE the work of Sanger (1) on insulin it has become evident that proteins are characterized by a rigidly determined sequence of amino acid residues (2) It is obvious that to synthesize such polymers the organism must possess a large store of information encoding the sequence of all the proteins that it is capable of making and this store of information must be replicated and passed on from one generation to the next

The chemical nature of the information carrying structure has recently been established In the case of two viruses—one plant (3 4) and one animal (5)—the ribonucleic acid (RNA) carries all the information that is needed to determine the virus protein Since there is a large body of evidence implicating RNA in the synthesis of proteins it does not appear farfetched to assign an information carrying function to RNA generally

The information encoded in RNA presumably in the form of specific sequences of nucleotides must in turn come from somewhere Two possibilities present themselves either RNA is self replicating or there is another substance which determines its structure As there is now some evidence that the amino acid sequence of proteins is genetically determined (2) it is plausible to assume that desoxyribonucleic (DNA) acid determines the structure of RNA acting in some manner as a template for its synthesis and that RNA is thus the mediator of information between nucleus and cytoplasm If this is the case the virtual absence of cytoplasmic inheritance seems to rule out the possibility that RNA could be self replicating However in the case of viruses, RNA does replicate itself It can scarcely be supposed that the uptake of a molecule of virus RNA by a cell activates some latent mechanism for its replication

for in that case one would expect that all the species of RNA in a cell would become self replicating which appears to be contrary to the evidence The RNA of a virus probably differs from cytoplasmic RNA mainly in sequence of nucleotides and it is presumably the sequence which makes one RNA self replicating and another not How sequence differences can produce such a result is not however, very obvious

Before discussing this further I wish to present some experimental evidence which I believe bears on the problem of the synthesis of RNA The work to be described was done in collaboration with Dr George Brawerman (6) Before our results were published, very similar experiments were described by Pardee and Prestidge (7) and by Gros and Gros (8) In view of the concordance of results from three laboratories the facts seem well established I take the responsibility however for the conclusions I shall draw from them

No matter how information is encoded in RNA, a chemical mechanism must exist for its transcription to protein The problem of the relation of the metabolism of RNA to that of protein has therefore attracted attention It has been known that if protein synthesis is prevented by lack of amino acids the synthesis of RNA is affected also (9) Studying the formation of adaptive enzymes in *Escherichia coli*, Pardee found that adaptation and by inference protein synthesis would not occur unless components of RNA (uracil ribose phosphate) were available to the organism (10) He concluded that the syntheses of RNA and protein were reactions chemically coupled to each other and that RNA is merely the inert by product of protein synthesis

This interpretation implies that there must exist a stoichiometry between the syntheses of these two polymers and to test this we studied RNA and protein formation in a lysine requiring yeast (6) RNA was depleted by growth in a phosphate deficient medium, and the cells were then transferred to a medium complete in all respects but containing varying amounts of lysine We found that the amount of both protein and RNA formed was proportional *to the amount of lysine added The number of residues of amino* acids incorporated into protein however bore no simple ratio to the number of nucleotides incorporated into RNA being approximately 15 1 (Fig 1) Thus although normally the two syntheses occur concomitantly there is no simple stoichiometry between them suggesting that the coupling is of a rather indirect nature

Under certain conditions coupling can be abolished This occurs in bacteria in the presence of chloramphenicol which suppresses the synthesis of protein but allows the syntheses of other cell components to proceed normally (11 12 13) We therefore tested the effect of chloramphenicol using a tryptophan requiring mutant of *E coli* As in the analogous case of yeast a tryptophan deficiency stops both RNA and protein synthesis If chloramphenicol is now added, protein synthesis remains nil but the synthesis of RNA resumes

This clearly shows that chloramphenicol not merely permits RNA synthesis but actively uncouples its synthesis from that of protein However if the only mode of action of chloramphenicol is suppression of protein synthesis why should it permit resumption of RNA synthesis by blocking a reaction—the synthesis of protein—which is already blocked by lack of an amino acid?

It occurred to us that the normal coupling of the syntheses of protein and RNA arises because both processes require amino acids one for building blocks the other perhaps for some catalytic function This supposition at first sight is contradicted by the results obtained with our tryptophan requiring mutant, since on addition of chloramphenicol RNA synthesis resumes even

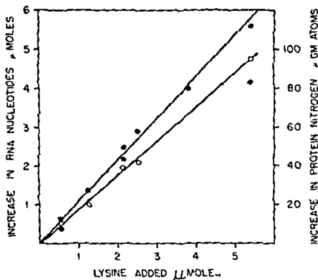


FIG 1 — Relation between amount of lysine added and quantity of protein and nucleic acid formed *Solid circles* ribonucleic acid *open circles* protein nitrogen From reference 6

in the absence of added tryptophan It is possible however that the mutant has available a small supply of tryptophan either because the genetic block is incomplete or through proteolysis Normally one might suppose tryptophan is swept up by the protein forming mechanism When protein synthesis is blocked by the addition of chloramphenicol small quantities of tryptophan might accumulate allowed RNA to be formed

A demonstration that an amino acid is indeed needed for RNA synthesis was obtained by using a histidine requiring mutant of *Aerobacter aerogenes* This organism besides requiring histidine for growth also possesses a powerful histidinase which presumably rapidly depletes its internal store of histidine On addition of chloramphenicol to histidine starved cells there is no rise

in RNA but, on further addition of histidine, RNA is rapidly formed (Fig 2) However, even with this organism these experiments were not an unqualified success Frequently, even after prolonged histidine starvation, on adding chloramphenicol a more or less significant rise in RNA would occur in the absence of added histidine In view of the results obtained by other workers, we feel reasonably confident that this is to be ascribed to the difficulty of completely depleting the cells of histidine Pardee and Prestidge (7) have demonstrated that several other amino acids are required for the synthesis of RNA

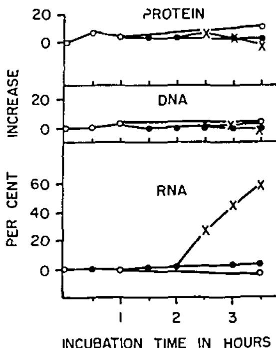


Fig 2 —Effect of chloramphenicol on a histidine deficient mutant of *Aerobacter aerogenes* *Open circles* in histidine free medium *solid circles* chloramphenicol added (after 1 hour) X histidine added (300 mg/l after 2 hours) From reference 6

in the presence of chloramphenicol Gros and Gros (8) have extended the list and in addition, show that the amounts of amino acids required are catalytic rather than stoichiometric It is probable that all twenty amino acids are required for the synthesis of RNA, although as yet this has not been rigorously shown In further discussion I shall assume that this is the case

There exists thus, a very interesting reciprocal relation between the syntheses of RNA and of protein Catalytic quantities of amino acids are required for the synthesis of RNA and, conversely the components of RNA are required for protein synthesis The synthesis of RNA is not dependent on the synthesis of protein Whether the synthesis of protein requires the simultaneous

synthesis of RNA is not known but such a requirement is unlikely in view of the obvious lack of stoichiometry of these two processes

I believe that these facts can be explained by a simple hypothesis Webster and Johnson (14) demonstrated that the synthesis of protein requires the nucleotides which occur as components of RNA This probably means that the precursors of proteins are some kind of compounds having the general structure of an amino acid linked to a nucleotide I would like to suggest that such compounds could well be the precursors likewise of RNA This hypothesis is schematically illustrated in Figure 3 Identical precursors are used by two types of templates The protein forming template lines up the precursors and acting catalytically polymerizes the amino acid portions the nucleotides being released The RNA forming template also acting (as experimentally demonstrated by Gros and Gros) catalytically polymerizes the nucleotide portion the amino acids being released There is thus no real coupling

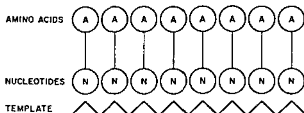


FIG 3 —Schematic representation of the synthesis of RNA and protein from common precursors (see text) The nature of the template is presumed to determine whether the aligned precursors polymerize to produce protein or RNA From reference 2

of protein and RNA syntheses the apparent coupling normally observed arising because both are formed from the same precursors When the supply of common precursors is depleted both syntheses must obviously stop

We may now consider the implications of this hypothesis for the replication of viral RNA As there are four kinds of nucleotides in RNA and as omission of any one of the twenty amino acids stops RNA synthesis even those precursors which bear the same nucleotide cannot be equivalent as far as the RNA forming mechanism is concerned The template which aligns the precursors must therefore distinguish twenty kinds of items although the finished product contains only four That this is analogous to the synthesis of proteins is rather surprising and probably significant

To replicate viral RNA one must translate a four symbol text (RNA) into itself The simplest way of doing this would be to postulate a one to one correlation in such a way that each symbol (nucleotide) in the text would determine the same symbol in the translation Using this system, any text can obviously be translated into itself However since there are not four

but twenty different precursors the text actually must first be translated into one of twenty different symbols, which gives an RNA text after removal of the characteristic amino acids (Fig 3) To determine twenty symbols, at least three nucleotides per symbol are needed As the number of nucleotides in the original and the translation must be equal, each precursor in the translation is determined by nucleotides which also participate in the determination of other precursors The sequence of the aligned precursors must therefore suffer certain limitations as not all precursors can stand next to each other[1] The limitations on sequence of the finished product RNA will however be less severe This is because different precursors bear the same nucleotide and therefore many different sequences of precursors can correspond to the same sequence of nucleotides What limitations if any, persist in the RNA will depend on the exact way in which the precursor sequence is encoded in the original text The coding system used cannot of course be determined from these considerations

Giving a coding system it will be readily seen that in general RNA texts will fall into two classes a larger one which translates into another non identical one and a restricted class which translates into itself On the hypothesis presented here the second class of texts is equated with the RNA of viruses

This hypothesis leads to a verifiable prediction which of course is its only justification The RNA of viruses has been shown as previously mentioned to determined the amino acid sequence of the virus protein Since only a restricted class of RNA sequences, under this hypothesis, can be translated into themselves one might expect that the virus protein determined by this class of RNA would be characterized by considerable restrictions on the permitted sequence of amino acid residues This would be in contrast to normal proteins where any sequence of residues can apparently occur (2) Unfortunately *at the moment the sequence of amino acid residues in virus* proteins is virtually unknown

I postulate here that cells generally have an active mechanism for synthesizing RNA using an RNA template and that the only activation of virus synthesis is the presentation to this mechanism of a suitable template the virus RNA Since genetic evidence appears to require that RNA should not be indefinitely self replicating it is pertinent to inquire what such a mechanism for replicating RNA might be doing in the uninfected cell Lack of experimental evidence prevents a definite answer to this *question* One suggestion could be as follows RNA like DNA may be a double stranded structure (17) If this is so it is possible that a DNA template synthesizes

1 That limitations on sequence must exist when a twenty symbol text is encoded into one of four symbols of the same length was pointed out by Gamow (15) For a detailed discussion of the point see Gamow *et al* (16)

a single strand of RNA (17) and that the postulated mechanism then proceeds to build up a complementary strand of RNA, which remains as part of a double stranded structure and undergoes no further replication Virus RNA one might then assume is incapable of forming firm double stranded structures because of peculiarities of nucleotide sequence permitting indefinite self replication Only more information on RNA structure and metabolism can determine whether this is a plausible suggestion or not

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CHAPTER 11

Relation of Oxidation, Phosphorylation, and Active Transport to the Structure of Mitochondria*

Albert L. Lehninger

THE functional relationships between the ultra structure of the mitochondrion and its very complex enzymatic activities offer some of the most challenging current problems in molecular biology, in which some interesting progress is being made. In this chapter some of the basic landmarks of these relationships are outlined, together with some consideration of the nature of the problems which must still be met in order to provide a molecular basis for the structure and physiologic function of these important cytoplasmic bodies.

A number of different experimental approaches and developments now give promise of revealing some of the very subtle and complex relationships between the structure and function of mitochondria. Among these are the increasing resolving power of the electron microscope as applied to ultra thin sectioned material; the study of the complex enzyme systems involved in respiration and phosphorylation by the classical methods of the enzymologist; the application of spectroscopic methods for analyzing the function of the respiratory carriers in intact mitochondria; the study of intra mitochondrial localization of various enzymes and enzyme systems; the recognition of the ability of mitochondria to cause movement of water and electrolytes coupled to respiration and phosphorylation; and the finding that thyroxine and its analogs react with some aspect of mitochondrial structure to cause alterations in structure and reactivity. It is striking that current study of the structure and function of mitochondria is employing the tools and outlook of the chemist, physicist, and morphologist to very great advantage, with increasing integration of these approaches.

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A SURVEY OF THE ENZYMATIC ACTIVITIES OF MITOCHONDRIA

With the perfection of the sucrose medium for preparing cell dispersions and for centrifugal isolation of mitochondria it has been possible to establish that mitochondria isolated from a large variety of cell types both animal and plant have organized respiratory and phosphorylating activity of such magnitude that it can easily account for the total respiration of the intact cell (1–4) In liver, kidney and heart the most frequently studied tissues the mitochondria catalyze the various reactions of the Krebs tricarboxylic acid cycle in a smooth and integrated fashion Electrons removed from intermediates of the cycle by action of dehydrogenases channel through the respiratory chain via pyridine nucleotides to the flavoprotein carrier and the cytochrome system Coupled to this process of electron transport is the phosphorylation of ADP at three distinct sites in the chain In fact the ability to catalyze oxidative phosphorylation has tacitly become a functional criterion for identification of mitochondria and the P O ratio of the coupled phosphorylation has become a means of assessing the degree of intactness of mitochondrial structure although it is perhaps not the most sensitive

In addition to these basic energy transforming mechanisms mitochondria from many cell types also contain the requisite enzymes for activation and oxidation of fatty acids to the stage of acetoacetate or more usually to CO_2 and H_2O via the formation of acetyl CoA the immediate fuel of the tricarboxylic acid cycle

These respiratory and energy transforming reactions appear to be the characteristic enzyme systems of mitochondria the possession of a respiratory chain with phosphate coupling mechanisms appears to be the most fundamental common denominator There are however a number of other single enzymatic activities found in the mitochondrial fraction which will not be remarked on here in any detail (see [5]) However it should be pointed out that some hydrolytic enzymes such as β glucuronidase ribonuclease deoxyribonuclease uricase and others which have been found in the mitochondrial fraction of some tissues may in fact be localized in another cytoplasmic structure which contaminates the mitochondrial fraction namely the so-called droplets or lysosomes (6)

Another type of metabolic function appears to be associated with the mitochondria although its scope of occurrence in different cell types remains to be fully explored active transport mechanisms associated with the movement of H_2O and certain electrolytes (see Davies [7]) Such mechanisms are coupled to respiration and phosphorylation which apparently furnish the driving force for these endergonic processes Although the physiological significance of these active transport activities is not yet entirely clear it appears mandatory to attach considerable importance to this phase of mitochondrial activity because

of the characteristic morphology of the mitochondrion which suggests a role in active transport by virtue of its compartmental membrane structure and also the compelling fact that the mitochondria are the major intracellular sites of transformation of respiratory energy which must in turn be the major driving force in aerobic cells for transport of materials against gradients of chemical potential

STRUCTURE OF MITOCHONDRIA

Following the pioneering electron microscopic investigations in the laboratories of Palade (8) and Sjostrand (9) the gross features of mitochondrial structure as seen in thin sectioned intact tissues are now reasonably well established These bodies which are about 1–4 μ long are composed of an outer insoluble double membrane surrounding an inner less dense matrix containing considerable soluble material including proteins enzymes nucleotides and electrolytes Extending inward from the inner surface of the membrane are baffles or incomplete septa the cristae mitochondriales There has been some difference of interpretation regarding the structural relationship of the cristae to the outer membrane Sjostrand has suggested that the outer membrane is double layered with no connection or continuity with the septa which also have double layer construction Palade on the other hand on the basis of careful examination of sections cut through different axes has made the very reasonable interpretation that the cristae actually represent invaginations of the inner layer of the double membrane surrounding the mitochondrion and that the double layer construction of the cristae is accounted for by adjacent and contiguous folds of the invaginated membrane This view appears now to be widely accepted and seems to account for most observations on the arrangement of the cristae and their response to damage or swelling of the mitochondria

The thickness of the mitochondrial membranes has been determined from electron micrographs It has been estimated that the double membrane at the periphery of the mitochondrion has a total thickness of about 160 A Sjostrand has suggested that this thickness may correspond to a double layer construction composed of two external monolayers of protein molecules each about 45 A thick separated by a double layer of oriented lipid moledules about 70 A thick Although it is known that the membrane is quite rich in lipid it is perhaps premature to conclude that lipid and protein are necessarily stratified in the manner suggested by Sjostrand although the double layer construction is certainly a striking feature in osmic acid–fixed sections If Palade s view on the structure is correct each of the two layers of the outer membrane has a thickness of about 80 A the inner one invaginating to form the cristae which thus also have a total thickness of about 160 A for a double fold It is of great

interest that the membranes may represent monolayers of lipoprotein mole cules since it is known that the membrane contains organized assemblies of electron transfer and phosphorylation enzymes most likely in some close packed geometrical array to permit easy interaction and electron transfer these are known to be rich in lipid

It is a very important fact that this basic structure and the dimensions of the outer double layer are constant and characteristic in all kinds of mitochon dria which have been studied even from very diverse cell types The constant finding of respiratory and phosphorylating activity and also of this character istic construction of the mitochondria in many different kinds of cells is thus a relationship of great comparative significance

Unfortunately the otherwise very revealing electron microscopic studies on fixed, non living material leave an impression that the mitochondrial structure is rather rigid and fixed Actually however they should be regarded as bodies with considerable plasticity of form and volume which is readily seen in un fixed cells particularly under phase contrast Although the structure of the membranes and cristae is characteristic and constant the shape and volume undergo constant change as the mitochondria move in the cytoplasm The probability that the mitochondrial membrane is reversibly contractile will be discussed below

Electron micrographs have revealed that mitochondria of rat liver and kid ney isolated from 0 25 *M* sucrose homogenates (the classical medium used in most studies) (5) are by no means intact and undamaged They are usually swollen and nearly spherical and the cristae are not easily visualized Efforts have been made to improve the isolation procedure Novikoff for example has isolated liver mitochondria from media containing both sucrose and polyvinyl pyrollidone which appear to be very close in morphology to those seen in sec tions of intact liver (10)

DEPENDENCE OF ENZYMATIC AND METABOLIC FUNCTIONS ON STRUCTURAL INTEGRITY

It has been appreciated for some years particularly by students of oxidative phosphorylation that there is a close interrelationship between respiratory activity and phosphorylation and the integrity of mitochondrial structure (1–4) Although mitochondria as usually isolated from 0 25 *M* sucrose are far from being morphologically intact any further degradation of their structure ordinarily leads to loss of enzymatic organization For instance the oxidation of fatty acids and pyruvate has been found to depend on the integrity of the mitochondria damage to the structure by freezing and thawing extreme hypo tonicity detergents or aging has been found to lead to disorganization of en zymatic activities so that only certain partial reactions of the Krebs cycle can

be observed Oxidative phosphorylation is even more sensitive these procedures usually lead to complete and essentially irreversible inactivation of the phosphorylation mechanisms The ability to carry out oxidative phosphorylation with a high P O quotient has thus been rather generally accepted as a criterion of intactness of structure However, this criterion is not now considered particularly sensitive since oxidative phosphorylation may actually occur with a normal P O quotient in swollen mitochondria Furthermore it has recently been possible as will be described to disrupt mitochondria rather completely into very small fragments which will still carry out oxidative phosphorylation

Perhaps a more sensitive functional criterion may now be established through tests of the mitochondria to carry out reactions of active transport because these reactions appear to be the most labile and evanescent of mitochondrial functions (7 11) Since mitochondria as usually prepared in 0 25 *M* sucrose show disorganization of the cristae as well as imperfect reproducibility of demonstrations of *H O and electrolyte transport the latter functions may be asso*ciated with the integrity of the cristae and may thus serve as a more critical functional criterion of structural integrity

INTRA MITOCHONDRIAL LOCALIZATION OF ENZYME ACTIVITIES

From a number of studies in different laboratories it is now possible to assign intra mitochondrial sites for certain enzymatic activities associated with mitochondria For example it has been known for many years through work of Warburg and later of Keilin and Hartree that the respiratory chain activity of the cell is associated with particulate elements from which it cannot be dissociated in soluble form More recently it has been concluded that the enzymes of the respiratory chain are actually associated with insoluble elements of the mitochondrial membrane for instance the classical Keilin Hartree heart muscle preparation of the cytochrome system has been established to represent fragments of the sarcosomal membrane (12) The most convincing evidence has come recently from studies of Watson and Siekevitz (13) who have combined enzyme assays with electron microscopic examination of submitochondrial preparations having respiratory activity such studies revealed clearly the characteristic structural features of the mitochondrial membrane in the insoluble fragments having respiratory activity which were recovered after dissolution of mitochondria with solutions of deoxycholate

There appears to be little or no organized respiratory chain activity in the soluble matrix in the interior of the mitochondrion On the other hand it may be presumed that many of the dehydrogenases and other enzymes acting on intermediates of the tricarboxylic acid cycle and the fatty acid oxidation cycle are found in the soluble material of the matrix since many of these enzymes

are easily extracted from disrupted mitochondria in soluble form (4 5) However not all the dehydrogenase proteins are present in soluble form Recent studies on membrane fragments (14 15) indicate that succinic dehydrogenase D β hydroxybutyric dehydrogenase and also transhydrogenase are associated with the membrane fragments ATPase activity is also associated very largely with the membranes (14) In addition it appears probable that the soluble matrix contains a number of nucleoside di and triphosphates together with enzymes capable of reversible transfer of phosphate from ATP to other nucleoside diphosphates

The consequences of the restriction of diffusion of the electron carrier proteins which is occasioned by their presence in the mitochondrial membrane have been given some consideration For instance Chance and Williams (16) have considered two degrees of restriction In one situation the molecules would be free to rotate about a fixed center affording some opportunity for restricted intermolecular collisions In the second case rotational and translational movement would be completely excluded by a rigid arrangement which would require that the passage of electrons from the prosthetic group of one carrier to that of the next in the chain must occur through the protein moieties in accordance with the idea of an electron continuum originally postulated by Szent Gyorgyi In any case restricted diffusion of these enzyme molecules would appear to impose some limitations on the treatment of the kinetics of electron transport and phosphorylation on the basis of classical mass law considerations which Chance and Williams have acknowledged although they point out that their observations of the kinetics of electron transport in intact mitochondria *in situ* as deduced from spectrophotometric measurements are apparently fully consistent with classical treatments based on collision theory

The fixed arrangement of respiratory carriers in the mitochondrial membrane in such an array or assembly with restriction of translational or rotational movement is almost a philosophical necessity since Ogston and Smithies (17) have pointed out that the rate of respiration via a sequence of protein carriers if they existed in an ideal dilute solution where successive collision steps would be limited by the relatively low rate of diffusion of protein molecules would be so low considering the number of protein protein interactions involved that the known high rates of respiration of tissues would simply be impossible without some device to direct interprotein collisions

The arrangement of the respiratory enzymes in such a structure also poses some problems in protein chemistry To date it has not been possible to bring into truly soluble form and purify such membrane enzymes as cytochromes b a and a_3 or for that matter the DPN linked D β hydroxybutyrate dehydrogenase of liver and kidney mitochondria These enzymes are very tightly bound and have resisted a wide armamentarium of methods for solubilization for many

years The nature of the forces holding together these respiratory assemblies are not yet understood and indeed, most methods for solubilization are purely empirical very little sophisticated rationale for 'solubilizing such enzymes is available Most of these empirical efforts are centered on the presence of lipid and presumably lipoprotein in the membrane It can be expected that solid state lipoproteins of this kind may be considerably more complex than the soluble lipoproteins of blood plasma the structure of which is little enough understood

It is possible that covalent linkages exist between the protein moieties of some of the enzymes of the respiratory assembly which would provide a protein continuum i e a complex protein common to two or three or more enzymatic activities Possibly such an arrangement may underlie the consistent failures which have been reported to separate these enzymatic activities from one another in truly soluble form It is obvious then that the structure of the mitochondrial membrane as well as of other biological membranes may require the development of a new dimension of protein chemistry with its own set of principles and experimental operations

PHOSPHORYLATING FRAGMENTS OF THE MITOCHONDRIAL MEMBRANE

Because the coupling of phosphorylation to electron transport is a very labile process and dependent on some aspects of mitochondrial structure this enzymatic process has been investigated only by relatively indirect approaches applied to reasonably intact mitochondria the classical approach of separation of the individual enzymes and in vitro reconstruction of the system have simply been impossible to apply It is easily possible to obtain fragments of mitochondrial membranes which exhibit electron transport from certain substrates to O by treating mitochondria with chemical agents such as cholate or by mechanical disruption however such fragments do not ordinarily carry out oxidative phosphorylation of ADP coupled to their respiration This fact indicates that essential factors involved in the coupling process are lost when the mitochondria are disrupted by these procedures

The bottleneck in the progress in elucidating the mechanism of oxidative phosphorylation has thus been the inability to observe it in enzyme preparations less complex than intact mitochondria In our laboratory we have carried out considerable work to devise a method of preparing mitochondrial membrane fragments which are still capable of coupling phosphorylation to electron transport as a necessary first step in approaching directly the mechanisms of phosphorylation This goal has been achieved (15 18 19) by treating rat liver mitochondria with cold digitonin solutions in which they undergo structural disruption By differential ultracentrifugation of the extracts obtained in the

way it is possible to obtain a fraction of water insoluble particles derived from the membrane which carry out not only electron transport from D β hydroxy butyrate to oxygen via DPN and the cytochrome system, but also the coupled phosphorylations with measured P O quotients as high as 2 8 but more usually between 1 7 and 2 4 All three phosphorylation sites appear to be functional in such preparations The different cytochromes are present in the same molar ratio to each other in such digitonin fragments as in intact mitochondria as revealed by difference spectrophotometry (15)

These phosphorylating fragments are not simply miniature replicas of intact mitochondria since they are no longer able to catalyze reactions of the tricar boxylic acid cycle or the fatty acid oxidation cycle From the e considerations it appears as though these particles contain complete respiratory chain as semblies, together with the enzymes necessary for coupling phosphorylation to electron transport but which are no longer connected to the enzymatic reac tions of the Krebs cycle and fatty acid cycle which presumably take place largely in the soluble matrix within the mitochondrial body

These phosphorylating digitonin fragments although polydisperse in a centrifugal field have a relatively low particle weight ranging from perhaps 3 000 000 to 40 000 000 *Significantly, it has been found that the enzymatic* and chemical constitution of these particles is constant per milligram total nitro gen *and independent of particle weight indicating that the particles of different* sizes are made up of identical recurring units (14) We have accordingly sug gested that the mitochondrial membranes and cristae contain many assem blies of respiratory chain enzymes evenly distributed over the membrane and that the assemblies are separated by labile lines of cleavage so that fragmen tation of the membrane leads to a series of pieces which are all multiples of a basic unit each containing a single respiratory phosphorylating assembly of perhaps a dozen separate enzyme proteins imbedded in a characteristic way in a lipoprotein fabric which not only provides floor space for the respiratory and phosphorylating machines but also as will be seen below controls entry and accumulation of metabolites

It is then of interest to make some speculations concerning the number of such respiratory assemblies found in the membrane of a single rat liver mito chondrion If certain assumptions are made regarding the number of enzymes making up an assembly the molecular weight of each enzyme and the average turnover number of each enzyme it can be calculated that each mitochondrion contains some thousands of these respiratory assemblies imbedded in its membrane Such calculations are of course only the crudest approximations but if they are anywhere near correct it appears possible that a substantial portion of the weight of the mitochondrial membrane could be made up of respiratory enzyme proteins possibly 10 per cent or more Such considerations

suggest that the membrane is alive, enzymatically speaking and not merely a dead wall between two compartments

The phosphorylating fragments just described have been most useful in approaching more closely the enzymatic mechanisms involved in oxidative phosphorylation and electron transport since they afford the opportunity of exploring these mechanisms directly without the necessity of including the complications introduced by other enzymatic reactions such as occur in intact mitochondria or in such derived phosphorylating preparations as described by Ziegler *et al* (20) which represent considerable degradation of mitochondrial structure but which still catalyze many of the reactions of the Krebs tricarboxylic acid cycle

ELECTROLYTE BINDING AND THE MITOCHONDRIAL MEMBRANE

It has been demonstrated that intact mitochondria when carrying out phosphorylating respiration show an increased rate of exchange of certain electrolytes or even an active accumulation against a gradient (7 11) Evidence has been obtained for the active transport of K^+ Na^+ Mg^{++}, and H^+ by rat liver mitochondria These reactions are rather sensitive to the ambient conditions and very little is known concerning the molecular mechanisms involved in active transport of such electrolytes and of the molecular preference of these mechanisms

Recent work in our laboratory by Gamble has opened up some new experimental approaches to the study of the mechanism of active transport of K^+ (21) It has been found that selective binding of K^+ coupled to phosphorylating electron transport can be observed to occur not only in intact mitochondria but also in the mitochondrial membrane fragments prepared from digitonin extracts which have been described above Such membrane fragments contain considerable bound K^+ when freshly prepared but very little bound Na^+ The bound K^+ is quickly lost on aging but is preserved if the particles are carrying out respiration The dynamics of the binding reaction can be followed with radioactive K^+ the binding of which is greatly enhanced by respiration The binding reaction is abolished by inhibiting respiration with cyanide or by poisoning phosphorylation with 2 4 dinitrophenol although the presence of phosphate or ADP is not required for the binding of K^+ Although Na^+ inhibits the exchange of K^+, it was found that this inhibition is not caused by replacement of K^+ at the binding sites by Na^+

These experiments provide not only further evidence of the high degree of enzymatic and functional interplay in the mitochondrial membrane and possibly a new membrane function but also an important experimental approach to the molecular basis for active transport of cations and for selectivity of transport mechanisms

WATER TRANSPORT IN MITOCHONDRIA

Considerable evidence is now available suggesting that water may move in and out of isolated mitochondria when tested in vitro in a manner which is dependent on the occurrence of phosphorylating respiration This property is consistent with the changes in mitochondrial volume and shape which can be observed in vivo Two phases in what appears to be a reversible process may be distinguished although it must be understood that these processes still require a great deal of further study first a swelling phase which appears to be passive in nature (i e not dependent on respiration) and in which water moves into the mitochondria and second a respiration coupled phase in which water may be actively extruded from mitochondria under certain experimental conditions It has been suggested by Raaflaub (22) and by Price *et al* (11) that these processes are best explained by the occurrence of a reversible respiration coupled contraction of the mitochondrial membrane as though it has actomyosin like properties

The passive swelling phase may be studied easily in vitro When mitochondria are suspended in isotonic sucrose solution (22–24) in the absence of metabolites nucleotides or respiration they swell spontaneously a process which can be measured by the increase in H_2O content or by the decrease in optical density of the suspension The per cent dry weight of the mitochondria may decline from well over 20 to less than 15 per cent during the swelling Such passive swelling is promoted by inorganic phosphate in low concentrations by Ca^{++} and by SH binding agents As will be described below this swelling phase is also greatly promoted by thyroxine and its analogs The swelling phase may also be inhibited ATP ADP Mg^{++} Mn^{++} and EDTA will partially or completely inhibit swelling Curiously 2 4 dinitrophenol (DNP) as well as certain other agents capable of uncoupling phosphorylation from respiration will also inhibit the passive swelling reaction (23) These properties are all quite reproducible and have been observed in a number of laboratories The mechanism of these effects is still obscure (as is also the mechanism of relaxation of the myofibril) It is possible that at least some of the effects are mediated through metal ions tightly bound to the membrane such as Mg^{++} However as will be described the passive swelling reaction is also highly dependent on the oxidation reduction state of the respiratory carriers in the mitochondrial membrane suggesting a rather subtle and complex molecular mechanism for controlling the rate and extent of entry of water and solutes

On the other hand the other phase—the active extrusion of H_2O from partially swollen mitochondria—coupled to respiration and phosphorylation is a more evanescent phenomenon which has been observed repeatedly but which depends on factors other than respiration and phosphorylation that are not yet fully understood The extrusion phase has been observed to occur when mito

chondria which have partially swollen spontaneously in the absence of substrates or nucleotides are now provided with the latter in the presence of oxygen (11) Concomitant with the restoration of phosphorylating oxidation is a decrease in H_2O content It is probable that the great lability of this active extrusion process is a function of the still imperfect methods available for isolating mitochondria It is interesting that DNP apparently prevents this active extrusion phase an action which appears to be different from the inhibition of swelling of mitochondria by this agent at least superficially From a comparison of the molar amounts of H_2O moved with the molar respiratory flux it would appear that far more H_2O is moved than ATP generated for instance and such observations form a basis for the suggestion that the extrusion of water may be the result of a contractile process in the membrane

The suggestion of a reversible contractile process in the membrane has other aspects which may be amenable to comparative study vis a vis the actomyosin system It is well known that fresh mitochondria have no ATPase activity but considerable latent ATPase activity may appear following swelling or treatment with 2 4 dinitrophenol It has recently been found that the ATPase activity of L myosin is also stimulated by 2 4 dinitrophenol (25) a fact which suggests a common denominator in the contractile mechanisms of the mitochondrion and myofibril which may be exploited experimentally

THE ACTION OF THYROXINE ON MITOCHONDRIA

Many investigators have examined in vitro effects of thyroxine and its analogs on various isolated enzymes and enzyme systems in the search for its mode of action and a number of inhibitory effects have been described The effect which has attracted most attention is the ability of thyroxine to uncouple oxidative phosphorylation and produce compensatory increases in respiration of suspensions of mitochondria (26) under special conditions However this in vitro uncoupling effect has been seen only with concentrations of thyroxine which are at least a thousand times greater than known to be present in the tissues furthermore inhibitory effects on oxidative phosphorylation of mitochondria isolated from the liver following treatment of normal rats with thyroxine are seen only after relatively enormous doses which are far beyond the physiological amounts required to produce moderate elevation of metabolic rates

It was pointed out above that fragments of the mitochondrial membrane can be isolated from digitonin extracts which still show oxidative phosphorylation Studies in our laboratory have revealed that there are two agents which uncouple phosphorylation in intact mitochondria but which do not inhibit phosphorylation at all with the isolated membrane fragments namely, Ca^{++} and thyroxine whereas DNP uncouples phosphorylation in the membrane

fragments with the same efficiency as in intact mitochondria (27) This finding clearly indicates that thyroxine has no direct effect on the enzymes or co factors involved in coupling phosphorylation to respiration but must exert its uncoupling action on intact mitochondria by indirect means

It appeared possible that thyroxine might act on the mitochondrial membrane in such a way as to cause some disruption or dislocation of the phosphorylating enzymes in the membrane fabric so that they are no longer functional but are not actually inhibited by direct action of the thyroxine Accordingly investigations of the effect of thyroxine on the mitochondrial swelling reaction (i e the "passive phase as described above) were carried out and it was soon observed (2 23) that thyroxine greatly increases the rate of swelling of rat liver mitochondria when suspended in isotonic sucrose at pH 7 4 this action occurred at concentrations considerably lower than those required to uncouple phosphorylation Of all the agents capable of increasing the rate of swelling of mitochondria L thyroxine and its analogs—triiodothyronine tetraiodothyroacetic and triiodothyroacetic acids—are by far the most potent After some refinement of test conditions it was possible to demonstrate the swelling action by 1×10^{-8} *M* thyroxine (28) which is approximately the concentration existing in the blood and tissues Curiously Ca^{++} has a similar action but of a far lower degree of activity On the other hand as was mentioned before 2 4 dinitrophenol inhibits the passive swelling indicating a quite different type of action which is a parallel to the case of the ATPase activity of L myosin which is stimulated by 2 4 dinitrophenol but is inhibited by thyroxine (29) The swelling action of thyroxine on mitochondria can be inhibited or prevented by ATP Mg^{++} or significantly 2 4 dinitrophenol

On studying the conditions of the thyroxine swelling reaction more closely (28) it was found to occur only when the mitochondria were relatively intact and capable of oxidative phosphorylation for instance in media of isotonic sucrose or KCl In distilled H_2O there was no action nor was any observed in already aged or swollen mitochondria It was also of interest that the swelling reaction had a very high temperature coefficient with a Q_{10} of approximately 4–5 many membrane reactions are characterized by high temperature coefficients

Parallel studies have been carried out with L thyroxine labeled with I^{131} to assist in determining the nature of the interaction of thyroxine and the membrane (30) It has been found that thyroxine is bound reversibly in a manner similar to the Michaelis relationship between an enzyme and an inhibitor with half maximal binding at an ambient thyroxine concentration of about 2×10^{-8} *M* The binding like the swelling reaction is dependent on pH and virtually ceases at pH 9 0 On the other hand the binding reaction has apparently a very low temperature coefficient suggesting that the swelling reaction is pre

ceded by a binding reaction in a diphasic process The bound thyroxine is removed from the mitochondria by washing or by treatment with serum albumin which binds thyroxine and thus competes with the mitochondria

From these studies it appears that uncoupling of phosphorylation may be an indirect and possibly toxic consequence of the action of thyroxine on the mitochondrial structure, since the swelling effect is easily demonstrable at concentrations of thyroxine which fail to uncouple phosphorylation Uncoupling of phosphorylation has been seen only with concentrations at least a thousand fold greater than those in the tissues and inhibition of phosphorylation in thyroxine treated rats requires massive doses far beyond those required to produce a moderate elevation of the metabolic rate On the other hand mitochondria isolated from hypo and hyperthyroid rats differ in their spontaneous swelling rate in the directions predicted from in vitro tests of thyroxine hyperthyroid mitochondria swell much more rapidly than normal, and hypothyroid less rapidly (23)

OXIDATION REDUCTION STATE OF THE RESPIRATORY CARRIERS AND MEMBRANE PROPERTIES

While investigating the action of thyroxine on mitochondrial swelling we became aware of a very important factor controlling the passive swelling phase namely the oxidation reduction state of the respiratory carriers in the mitochondrial membrane (31) It was found that thyroxine would cause swelling of rat liver mitochondria when they were aerobic in the absence of added substrate On the other hand making them anaerobic or adding cyanide to inhibit cytochrome oxidase completely abolished the swelling action of thyroxine as well as the much smaller spontaneous swelling From appropriate spectrophotometric measurements it was found that this inhibition is associated with a change in the dynamic steady state of the respiratory carriers Under aerobic circumstances even with no substrate present a substantial fraction of the carriers is in the oxidized state When oxygen is excluded or when cytochrome a_3 is inhibited with cyanide the carriers become reduced at the expense of endogenous or exogenous substrates, and under these circumstances no swelling is produced by thyroxine

These observations are considered to be highly significant with respect to the intracellular control of the movement of water and electrolytes into and out of the mitochondria Under normal circumstances in the cell, mitochondria are supplied with both substrates oxygen phosphate and ADP, and respiration occurs However the oxidation reduction state of the respiratory carriers is a resultant of the relative rates of reduction or oxidation of the different carrier Chance and his colleagues (16) have demonstrated that, in liver mitochondria with ample oxygen substrates, and ADP, those carriers near the substrate (i e,

DPN and flavoprotein) are relatively reduced and those near oxygen such as cytochrome a and a_3 are relatively oxidized Thus the oxidation reduction state of the carriers in intact respiring mitochondria is poised in such a way that it could be a very important factor controlling the receptivity or sensitivity of the mitochondria to thyroxine or, indeed, other swelling agents such as ortho phosphate the rate and direction of movement of water and solutes would thus be under a very delicate control by the oxidation reduction state of the respiratory carriers

This action of the oxidation reduction state on the thyroxine effect has an other correlate Mitochondria catalyze an exchange reaction between phos phate labeled with P^3 and the terminal phosphate of ATP in the absence of respiration a reaction which is believed to reflect the action of a portion of the enzymatic mechanism linking phosphorylation to electron tran port This re action has now also been found to be dependent on the oxidation reduction state of the respiratory chain it is maximal when the chain is in the oxidized state and at a minimum when the chain is reduced (52)

From such observations it may be suggested that the contractile and relaxing powers of the mitochondrial membrane are conditioned by the oxidation reduc tion state of the respiratory enzyme assemblies which are present in the mem brane and which, in fact, may make up a significant part of its substance It is in this relationship that some of the secrets of the molecular mechanisms of active transport must be found here also may be found the molecular basis for the physiological action of thyroxine

From all these considerations it is evident that the enzymatic and molecular organization of the mitochondrion is a matter of considerable complexity with very subtle and intricate interrelationships between structure and function From experimental approaches of the kind described in this chapter it may eventually be possible to speculate on the interesting question of the biological advantage or necessity of the characteristic morphology of the mitochondrion

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CHAPTER 12

Enzyme Models*

Jui H. Wang

ENZYMES constitute a special class among all catalysts not only in their specificity but also in their outstanding catalytic efficiency. Molecule for molecule, there is no synthetic catalyst that can compete or even get anywhere near the enzymes in catalytic efficiency.

Finding out just how enzymes can catalyze so efficiently is certainly a problem of basic scientific importance. During the last fifty years a huge amount of valuable work has been carried out on enzymes. But up to now no one has yet succeeded in elucidating the structure of any one enzyme or in finding out just exactly how any enzyme performs its function. In some cases the enzymic activity can be attributed to certain chemical components called "coenzymes." Investigations on coenzyme-catalyzed reactions have yielded much valuable information regarding the mechanism of enzyme action. For example, the investigations on metal ion-catalyzed decarboxylation of oxaloacetic acid and related compounds by Krebs (1) and by Steinberger and Westheimer (2), the studies on the decarboxylation and transamination reaction catalyzed by pyridoxal carried out by Snell and co-workers (3), the work of Breslow (4) and others on the mechanism of thiazolium salt-catalyzed condensation reactions, etc., have substantially widened our understanding of the nature of enzyme action.

Unfortunately, coenzymes are much less efficient catalysts than the natural enzymes. Furthermore, only in special cases can an active coenzyme be isolated and studied. In general, the structure of the active center in an enzyme is unknown. Thus the major difficulty of this problem seems to be due to our lack of knowledge of the detailed structure of enzyme molecules, for although we have during the last decade learned a great deal about the basic structure of many proteins, we still know very little about the detailed structure of enzymes.

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While the valuable research on natural enzymes and coenzymes is being continued a number of workers have recently tried to approach this important problem of enzyme action from a new angle, i e by designing synthesizing and studying simple model molecules with possible enzyme like action Here I shall discuss only a few enzyme models to illustrate this method of approach rather than try to give an extensive review of the existing literature on this subject

MODELS BASED ON PURE ACTIVATION ENTROPY EFFECT

According to one school of thought, the major factor which is responsible for the remarkable efficiency of enzymes is the probability or entropy factor Ordinary reactions become slow at low reactant concentrations, because the collision frequency between the reacting molecules decreases as the concentration decreases But, for enzyme reactions, the substrate molecule is often bound right next to the catalyzing group until reaction takes place In other words in ordinary catalysis the activation step in the reaction involves a considerable loss of total entropy In enzyme reactions the activation step does not involve such an entropy loss because the substrate molecule is already bound firmly right next to the catalyzing group before the activation takes place Thus, according to this picture the advantage of an enzyme relative to a simple catalyst molecule should disappear at very high substrate concentrations

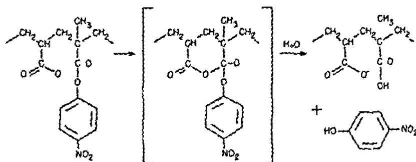
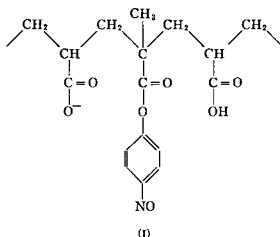


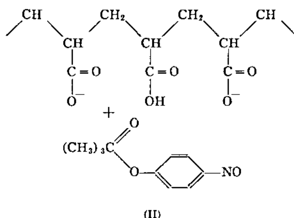
Fig 1

A direct experimental test of this prediction is unfortunately not possible because complications arise at very high substrate concentrations such as the change in dielectric constant of the solution, the large variation in the activity coefficients of the reactants etc However it is possible to evaluate this entropy effect by kinetic studies on compounds with catalyzing and substrate groups present in the same molecule For example, Morawetz and Zimmering (5) showed that the hydrolysis of the co polymer of acrylic acid and *p* nitrophenyl methacrylate (I) was several orders of magnitude faster

than that of *p* nitrophenyl trimethylacetate in a solution containing equivalent amounts of polyacrylic acid (II) at the same pH



(I)



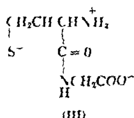
(II)

They suggested that the rapid solvolysis of the co polymer was due to intra molecular catalysis through the formation of a six membered cyclic inter mediate as represented in Figure 1

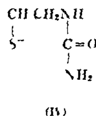
Recently Bernhard (6) discovered that the methyl ester of histidine is an extremely efficient catalyst for the hydrolysis for *p* nitrophenyl acetate De tailed rate studies showed that the reaction obeyed Michaelis Menten kinetics the methyl ester of histidine and *p* nitrophenyl acetate first formed an addition complex which subsequently decomposed into products and regenerated the catalyst The first order rate constant for the decomposition of this addition complex was found to be of the same order of magnitude as for the correspond

ing addition complex formed between chymotrypsin and p nitrophenyl acetate On the other hand the methyl ester of histidine is almost inactive toward normal esters (such as ethyl acetate) amides and peptides Thus it appears that the success of the above model systems might be due to the inherent instability of substituted phenyl esters The work of Bender and Turnquest (7) and of Bruice and Schmir (8) is suggestive of this possibility It is possible that in the catalytic hydrolysis of a normal ester amide, or peptide such a simple activation entropy effect is insufficient to account for the observed efficiency of hydrolytic enzymes

Attempts to construct model systems for the catalytic hydrolysis of normal amides and peptides have often yielded negative results For example compounds III and IV were synthesized in the writer s laboratory (9) as possible



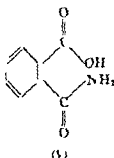
(III)



(IV)

model systems for the intra molecular mercaptide catalyzed hydrolysis of peptide and substituted urea Both III and IV were found to be quite stable in aqueous solutions at room temperature This shows either that the catalyzing groups in papain and urease respectively are not the simple sulfhydryl groups or that the activation entropy effect is only a small factor in the whole story about the efficiency of enzymes

Bender (10) recently made the interesting discovery that the hydrolysis of phthalamic acid (V) involves intra molecular acid base catalysis Kinetic measurements at different pH values showed that the rate of hydrolysis depends on the concentration of the undissociated phthalamic acid but is not proportional to the external hydrogen ion concentration These results suggest that the hydrolysis takes place through a direct intra molecular rearrangement



(V)

of the undissociated phthalamic acid The observed first order rate constant of this model system is however still much smaller than that for hydrolytic enzymes For example the first order rate constant for the hydrolysis of phthal amic acid was found by Bender to be 2×10^{-5} sec $^{-1}$ at 25° C, whereas that for enzyme catalyzed hydrolysis of amides is often in the range 1–10 sec $^{-1}$ at the optimum pH and the same temperature

MODELS BASED ON MOLECULAR INTERACTIONS

Quite aside from the *activation entropy effect* a number of theoretical models were proposed to account for the lowering of activation energy in enzyme reactions through interactions in terms of electric and dispersion forces between the bound substrate molecule and the protein part of the enzyme Thus in the "strained molecule theory proposed by Quastel (11) it was assumed that the substrate molecule does not fit perfectly but was strained and thus rendered more reactive Recently Kirkwood (12) showed that if the active center substrate complex possesses a larger dipole moment in the transi tion state than in the normal state one may expect the normal activation energy to be effectively lowered by favorable distribution of the average and fluctuating charges on the rest of the enzyme molecule Because of obvious experimental difficulties no one has succeeded in synthesizing a model molecule to test such a charge interaction theory of enzyme action Neither has reliable theoretical computation been made because of the uncertainties introduced in estimating the effective dielectric constant the dipole moment of the ac tivated complex, etc

MODELS BASED ON SIMULTANEOUS ACID BASE CATALYSIS

The rapid growth in popularity of these models appears to be encouraged by the remarkable work of Swain and Brown (13) They found that the muta rotation of tetramethylglucose in benzene solution was catalyzed by mixtures of phenol and pyridine But 2 hydroxypyridine which is a weaker acid than phenol and a much weaker base than pyridine was found to be a very much more effective catalyst than an equivalent mixture of phenol and pyridine For example the observed muta rotation rate of 0 1 *M* sugar in benzene solution is seven thousand times higher with 0 001 *M* 2 hydroxypyridine as catalyst than with 0 001 *M* phenol + 0 001 *M* pyridine as catalyst Kinetic studies suggested that 2 hydroxypyridine in this system is acting as a bifunc tional catalyst the acid and basic groups acting simultaneously on the sub strate

Analogous models were postulated for hydrolytic enzymes For example Barnard and Laidler (14) proposed an enzyme model in which two chemically independent but sterically juxtaposed acid and basic groups exert a concerted

push pull action on the bound substrate molecule Other workers later introduced slight modifications of their model but retained the basic idea of bifunctional acid base catalysis, as illustrated in Figure 2, where $-B$ represents the basic group and $-AH$ represents the acid group at the active site

However since the acid group in the enzyme is in rapid protolytic equilibrium with the surrounding water molecules the free-energy change in the activation step when the bound substrate molecule is protonated is the same, no matter whether the proton is donated by the acid group on the enzyme or by water molecules in its immediate neighborhood In other words as far as the protonated transition state is concerned there is even no need for the enzyme to

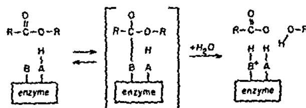


Fig 2

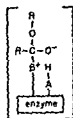


Fig 3

have such an acid group Consequently, except in those special cases where a metal ion is used as the acid instead of the proton the postulated existence of such an acid group in addition to the usual basic group is entirely superfluous

One may argue that the function of the acid group is perhaps not primarily to supply the proton but rather to stabilize the transition state by charge interaction or hydrogen bonding as indicated in Figure 3 This is quite reasonable But then the mechanism is no longer based on the simultaneous acid and base catalysis but on the combined effect of simple base catalysis and charge interaction discussed above

It is true that the valuable work of Swain and Brown on the mutarotation of tetramethylglucose in benzene solution shows the advantage of having an acid group in juxtaposition with a basic group for carrying out concerted action,

but the advantage in their case is due to quite different reasons In the first place the acid group in 2 hydroxypyridine is indispensable as the sole proton donor, since their substrate molecule was surrounded not by water but by benzene molecules Moreover since the catalysis by 2 hydroxypyridine on the one hand and that by phenol + pyridine on the other follow different kinetics the rate constants are in different dimensions and hence not directly comparable It is quite possible that the observed much faster rate in 0 001 *M* 2 hydroxypyridine solution as compared to 0 001 *M* phenol + 0 001 *M* pyridine solution is essentially due to an activation entropy effect Actually the two functional groups in 2 hydroxypyridine are not chemically independent of each other but are functionally linked through the conjugated double bond system Thus as soon as the basic group begins its attack on the proton of the hydroxyl group of tetramethylglucose the acid group becomes a much stronger acid than it originally was and can attack the ether O atom of the substrate concertedly and vice versa But this favorable effect of mutually reinforced attack should be assessed together with the unfavorable effect of the diminished acid and basic strengths of the two functional groups in 2 hydroxypyridine as compared to phenol and pyridine respectively

A SYNTHETIC CATALYST WITH CATALASE LIKE ACTIVITY

Because of the frustrating complexity of the structure of enzymes several workers recently turned their attention to the structure of the simple substrate molecules By making use of our knowledge of the structure of the substrate molecules the properties of the corresponding enzyme and the nature of the chemical bond sometimes small model molecules with enzyme like activity can be designed synthesized and studied It is possible that the detailed mode of action of such a synthetic catalyst may not be exactly the same as that of the corresponding enzyme in nature but experience indicates that the information obtained from separate investigations on related problems can often be very helpful As an illustration we shall discuss in considerable detail a synthetic catalyst for the decomposition of hydrogen peroxide first reported by the present writer i e the chelate compound formed by combining triethylenetetramine (TETA) $H_2NCH_2CH_2NHCH_2CH_2NHCH_2CH_2NH_2$ with ferric ion (15 16)

The decomposition of hydrogen peroxide is catalyzed by a large variety of substances But molecule for molecule none can compete with catalases for catalytic efficiency It is well known that catalases are iron enzymes with their iron in the ferric state But according to the estimates of Haldane (17) catalases are 10^9 times more efficient than simple ferric ion and 10^7 times more efficient than hematin or methemoglobin in catalytic action While examining the possible ways in which the simple ferric ion could be made

a more efficient catalyst for the decomposition of hydrogen peroxide it was speculated that perhaps the chelate compound formed between TETA and ferric ion could be a very good catalyst, for the following reasons Triethylenetetramine forms a quadridentate chelate compound with ferric ion Steric considerations show that it is energetically improbable to have the four N atoms and the ferric ion located in one plane, but that the structure with one primary amino N atom above and the other below the plane determined by the two secondary amino N atoms and the ferric ion is stable In this chelate compound the four N atoms form four co ordination bonds with Fe(III) along four

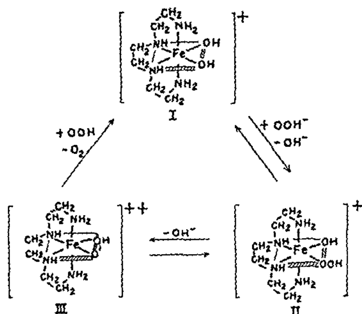


Fig 4

of the six octahedral hybrid atomic orbitals of the latter In aqueous solutions the two remaining octahedral orbitals are presumably used to bind two water molecules or hydroxide ions as illustrated by structure *I*, (TETA) *Fe(III)(OH)$_2^+$* in Figure 4 In the presence of hydrogen peroxide either or both of these unidentate ligands may be displaced by the OOH^- ions Since each OOH^- ion is potentially capable of forming two co ordination links with the Fe(III) ion the two attached OH^- ions in compound *I* of Figure 4 may be displaced by a single OOH^- ion yielding compound *III* But since the O–O bond length in OOH^- is only about 1 5 A, the O–O bond in compound *III* is under strain and hence rendered more reactive Direct splitting of the O–O bond in an isolated H_2O_2 molecule requires about 35 kcal of activation energy per mole But in the above mechanism the energy consumed in splitting the O–O

bond is partially compensated for by the energy gained through the formation of more stable Fe O bonds because the O atom and the OH^- ion are now separate ligands and can orient themselves freely for maximum overlap with the two vacant octahedral d sp^3 hybrid atomic orbitals of the Fe(III) Compound *III* can then readily react with a second OOH^- ion to yield O and regenerate compound *I* This cyclic mechanism was subsequently substantiated by experimental measurements on the rate of catalytic decomposition of H O by $(TETA)Fe(OH)_2^+$ The rates of catalytic decomposition of H O were measured both by direct titration and by manometric method with consistent results The turnover number of $(TETA)Fe(OH)_2^+$ expressed in moles of H O decomposed per minute per mole of $(TETA)Fe(OH)^+$ were computed from the observed initial rate of decomposition of H O Some sample data obtained with 0 15 *M* H O solution are listed in Table 1 In order to correct for traces of iron present

TABLE 1

CATALYTIC DECOMPOSITION OF H O BY $(TETA)FE(OH)_2$

C t t f T t l TETA (M l L)	C t t f T t l F (III) (M l L)	pH	T mp t (C)	T mb M
$2\ 9\times10^{-}$	$3\ 8\times10^{-}$	9 5	25 2	11 000
$2\ 9\times10^{-}$	$5\ 7\times10$	9 6	25 2	11 000
$2\ 9\times10^{-4}$	$7\ 6\times10^{-}$	9 6	25 3	11 000
$2\ 9\times10^{-}$	$3\ 8\times10^{-}$	9	1 8	4 400
$2\ 9\times10^{-}$	$3\ 8\times10^{-}$	9 5	13	6 700
$2\ 9\times10$	$3\ 8\times10^{-}$	9 5	41 5	21 000

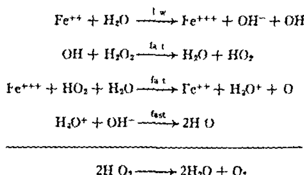
in the system as an impurity (from reagents and glassware) a blank measurement with TETA and H O but without added ferric ion was made at each temperature pH and substrate concentration These blank rates were subtracted from the corresponding measured rates before the computations for the turnover numbers were made The activation energy computed from Table 1 is 6 6 kcal mole Although $(TETA)Fe(OH)^+$ is not so efficient as natural catala es these data show that it is possible to construct small molecules with turnover numbers in the enzymic range

Kinetic measurements at different substrate concentrations showed that the decomposition rate continued to increase with H_2O concentration although the observed increase is slightly less than that predicted by a rate law that is first order with respect to H O concentration This shows that the transition from compound *II* to compound *III* (Fig 4) cannot be the slowest and hence the rate determining step in the catalytic cycle for in that case a Michaelis Menten type of saturation curve would be observed The rate determining step in the above catalytic cycle is either the reaction of compound *I* with

OOH^- to form compound *II* or the reaction of compound *III* with a second OOH^- to form O and regenerate the catalyst

Although the actual mechanism of catalase action may differ considerably from this synthetic catalyst detailed studies on the latter may be helpful in investigations on catalase itself Both $(TETA)Fe(OH)_2^+$ and catalase contain Fe(III) and both are inhibited by cyanide ions Regarding the relative catalytic efficiencies, this synthetic catalyst is still much inferior to natural catalases Thus by plotting the observed turnover numbers versus the molal concentration of H_2O_2 the second order rate constant k_2 (defined by the relationship $-d[H_2O_2]/dt = k_2[(TETA)Fe(OH)_2^+][H_2O_2]$) in dilute H_2O_2 solutions at 25° C and pH 10 may be estimated to be about 1.2×10^3 liter sec^{-1} mole^{-1} The corresponding value for catalase is about 1.3×10^8 liter sec^{-1} mole of hematin iron (18) Thus this synthetic catalyst is still inferior to catalases in efficiency by a factor of about 10^5 On the other hand it is superior to hemoglobin or hematin by a factor of about 10^4

Of course, the fact that the catalyst, designed with the expectation of fitting the cyclic mechanism postulated in Figure 4, is highly efficient does not prove that the postulated mechanism is correct The happy outcome of the experiments could be due to some kind of fortuitous coincidence For example, Haber and Weiss proposed early in 1932 (19) the following free radical mechanism for the catalytic decomposition of H_2O_2 by the Fe(III)–Fe(II) couple



$$Fe^{++} + H_2O_2 \xrightarrow{slow} Fe^{+++} + OH^- + OH$$

$$OH + H_2O_2 \xrightarrow{fast} H_2O + HO_2$$

$$Fe^{+++} + HO_2 + H_2O \xrightarrow{fast} Fe^{++} + H_3O^+ + O_2$$

$$H_3O^+ + OH^- \xrightarrow{fast} 2H_2O$$

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

The validity of the Haber Weiss mechanism is demonstrated by the observed initiation of free radical polymerization by the above mixture (Fenton's reagent) Thus one may ask whether the catalytic decomposition of H_2O_2 by $(TETA)Fe(OH)_2^+$ or catalases may also take place through the free radical mechanism This is very unlikely, for the catalytic efficiency of (TETA) Fe $(OH)^+$ is many orders of magnitude higher than the Fe(II)–Fe(III) system which is known to catalyze through the free radical mechanism Furthermore O^{18} isotope effect studies on the catalytic decomposition of H_2O_2 by either

ionic iron or methemoglobin indicated that the O^{18} enrichment factor for the Haber Weiss mechanism is 1 025 ± 0 003 (16 20) But in the case of catalysis by $(TETA)Fe(OH)^{+}$ (21) or by catalase (16 20) the O^{18} enrichment in the liberated O_2 is much smaller

A more convincing piece of evidence for ruling out the free radical mechanism in the catalysis by $(TETA)Fe(OH)_2^{+}$ is supplied by the observation that the chelate compound formed between tetraethylenepentamine (TEPA) $H_2NCH_2CH_2NHCH_2CH_2NHCH_2CH_2NHCH_2CH_2NH_2$ and ferric ion is practically inactive as compared to $(TETA)Fe(OH)_2^{+}$ At moderate catalyst and H_2O_2 concentrations the decomposition by $(TETA)Fe(OH)_2^{+}$ takes place with bursting violence, whereas a mixture of H_2O_2 and $(TEPA)Fe(OH)^{++}$ at the same concentrations may stand at room temperature for hours with little decomposition It may be recalled that the cyclic mechanism in Figure 4 is made possible by the two vacant adjacent octahedral atomic orbitals of the Fe(III) which may be occupied by the two pairs of co ordinating electrons from the same OOH^{-} ion and thus exert the splitting action Now in $(TEPA)Fe(OH)^{++}$ one of these orbitals is occupied by the fifth N atom of the ligand and hence the splitting mechanism is no longer possible i e the fifth N atom acts as an intra molecular inhibitor to prevent the above described splitting mechanism from taking place However the free radical mechanism is still possible with $(TEPA)Fe(OH)^{++}$ because the exchange of the electron can still take place through the sixth remaining orbital even though the rate may now be smaller by a factor of 2 Consequently the observation that $(TEPA)Fe(OH)^{++}$ is practically inert as compared to $(TETA)Fe(OH)_2^{+}$ shows that the catalytic decomposition by $(TETA)Fe(OH)_2^{+}$ takes place essentially through the splitting mechanism even though free radicals might also be present in the system

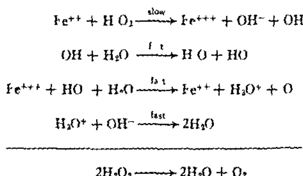
Replacing the TETA in $(TETA)Fe(OH)_2^{+}$ by other negatively charged ligands of similar structure often results in a chelate of little catalytic activity These ligands include *nitrilotriacetate ion* $N(CH_2COO^{-})_3$ *ethylenediamine* diacetate ion $^{-}OOCCH_2NHCH_2CH_2NHCH_2COO^{-}$ etc Presumably this decrease in catalytic activity is due to the increase in the ionic character of the Fe(III) ligand bonds in these chelates The cyclic mechanism in Figure 4 is based essentially on the directional character of the covalent bonds A purely ionic Fe(III) chelate would have little tendency to decompose H_2O_2 by the above splitting mechanism and hence would catalyze mainly through the much less efficient free radical mechanism

A rather extensive study was carried out on the catalase like activity of the chelate formed by TETA and various metal ions The metal ions selected for this study included Mg(II) Al(III) Ca(II) Cr(III) Mn(II) Fe(III) Co(III) Ni(II) Cu(II) Zn(II) Sr(II) Ag(I) Cd(II) Ba(II), Tl(I) and Pb(II) Of these, only the TETA chelate of Fe(III) and Mn(II) showed remarkable

OOH^- to form compound *II* or the reaction of compound *III* with a second OOH^- to form O_2 and regenerate the catalyst

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the O atom in compound *III* may remove a hydride ion from the OOH^- ion to form OH^- and liberate an O molecule

In order to determine the mechanism in this last step experiments were carried out with doubly O^{18} labeled hydrogen peroxide $HO^{18}O^{18}H$ The results are summarized in Table 2 When H O is made directly from O^{18} enriched water the relative abundance of the various molecular species $HO^{16}O^{16}H$ $HO^{16}O^{18}H$, and $HO^{18}O^{18}H$ *should be in accordance with that predicted by pure* probability considerations i e natural distribution (Here it is assumed that the isotope effect due to the difference in zero point energies of light and heavy O atoms in H_2O is negligible) Thus if the raw material used is water containing 10 78 atom per cent of O^{18} the mole per cent of $HO^{18}O^{18}H$ will be 10 78 $\times$ 10 78 = 1 16 When this H_2O is catalytically decomposed the mole per cent of $O^{18}O^{18}$ in the O produced should also be approximately 1 16

TABLF 2

T T L Atom P Ce o O in H O	M P C nt o HO 8O H D tf mi ed Oxida on with C (IV)	Mo P C o O O n O Li te C ti D m ositi n		
		P d t d f m A	P d t d f m B	E p m t ilv F d (21)
10 78	1 18	1 16	1 18	1 19 [(TETA)Fe(OH)]
5 20	0 559	0 270	0 559	0 556[(TETA)Fe(OH)]
5 20	0 559	0 2/0	0 559	0 54/[(TFTA)Fe(OH)]
5 23	0 563	0 274	0 563	0 556[Catalase]
5 21	0 566	0 271	0 566	0 547[Catalase]

no matter whether the decomposition takes place through mechanism *A* or mechanism *B* because the isotopic distribution in the original H O is natural This is verified by the first row of values in Table 2 The values 1 16 1 18 and 1 19 are equal within experimental errors However when this H_2O containing 10 78 atom per cent of O^{18} was mixed with approximately equal molal amounts of ordinary H_2O the total atom per cent of O^{18} and the mole per cent of $HO^{18}O^{18}H$ in this mixed H O became 5 20 and 0 559 respectively This last isotope distribution is unnatural for had water containing 5 20 atom per cent O^{18} been used directly as raw material the mole per cent of $HO^{18}O^{18}H$ in the resulting H O would have been equal to (5 20) / 100 = 0 270 Now if the hydrogen peroxide containing 5 20 atom per cent O^{18} and 0 559 mole per cent $HO^{18}O^{18}H$ is catalytically decomposed by $(TETA)Fe(OH)_2^+$ the mole per cent of $O^{18}O^{18}$ in the liberated O will be different according to whether the O is liberated through mechanism *A* or mechanism *B* Mechanism *A* involves a reshuffling of the O atoms i e restoration to the natural distribution of the isotopic species, and hence predicts a mole per cent of

0 270 for $O^{18}O^{18}$ in the liberated O_2 gas But mechanism *B* leaves the original unnatural distribution of oxygen isotopes undisturbed and hence predicts the mole per cent of $O^{18}O^{18}$ to be 0 559 With $(TETA)Fe(OH)_2^+$ as catalyst the observed mole percentages of $O^{18}O^{18}$ found in two separate experiments are 0 556 and 0 547 respectively Thus mechanism *A* is definitely ruled out Similar studies were made on catalase itself The results listed in the last two rows of Table 2, show that, in the catalytic decomposition of H_2O_2 by catalase the oxygen gas is also liberated through a hydride ion removal mechanism

The following question may be raised Is it possible to introduce modification in the structure of $(TETA)Fe(OH)_2^+$ so that a synthetic catalyst with even greater efficiency can be constructed? As a preliminary step to find the answer, we should first find out which step in the cyclic mechanism depicted in Figure 4 is the slow and hence rate determining one (22) Although the ligand replacement reaction which changes compound *I* to compound *II* in Figure 4 is expected to be fast by ordinary standards it may not be fast enough This is because we are here dealing with turnover numbers of the order of 10^4 min $^{-1}$ any replacement reaction with a half life longer than a millisecond is considered to be slow

On the other hand one may assume that the reaction between compound *III* in Figure 4 with a second OOH^- ion is the slow step If this is true one could design a more efficient catalyst in which the second OOH^- ion is bound right next to the active O atom in compound *III* for them to react In practice however the synthetic problem is often far from easy In fact the case of $(TETA)Fe(OH)_2^+$ is particularly fortuitous, because here the ligand field perturbed Fe(III) serves as both the binding and the catalyzing group In general, the binding and the catalyzing groups may have to be different We are then confronted with the difficult task of synthesizing model molecules with the binding groups so placed that they can hold the substrate molecule in exactly the right position for the catalyzing group to attack

Indeed, when one tries repeatedly one synthesis after another without success one just cannot help admiring how clever the designer of natural enzymes is in making use of proteins For in a sense each of these giant protein molecules is like a piece of wet clay which can be molded into innumerable shapes, holding the binding and catalyzing groups in exactly the right position and at the right orientation It is true that the natural way of folding the polypeptide chains in an enzyme molecule is not thermodynamically stable as is shown by the ease of its denaturation But the number of secondary bonds involved in the folding is usually large enough to make the activation energy for its denaturation much greater than kT, so that, once the enzyme molecule is folded in a characteristic way when it is made it will stay in that way

for quite a while, performing its marvelous function Obviously to synthesize enzyme models without making use of proteins is not going to be easy although it still seems worth trying

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CHAPTER 13

Lysogeny and Lysogenization—Studies in Infectious Heredity*

Salvador E Luria

LYSOGENY AND INFECTIOUS HEREDITY

TRANSFER of genetic properties among bacteria takes place by various mechanisms whose common characteristic is that they often resemble infection rather than sexuality if by infection we mean the occasional penetration and multiplication of an external agent into a living organism and by sexuality we mean the orderly merger of two complete sets of nuclear genes to give rise to a diploid (or polyploid) nucleus The transfer phenomena observed in bacteria include transformation transduction by bacteriophage and mating they have been exhaustively reviewed both in themselves (16 18 23 41) and in relation to phenomena of infection (22) The essential feature of these phenomena—the partial contribution of genetic material by a donor cell to a recipient cell—has been expressed by their collective designation as meromyxis (41)

The phenomenon of lysogeny (6 32) bridges the gap between the other examples of meromyxis and purely infective phenomena As a phage becomes reduced to prophage following infection of a susceptible cell we observe the transformation of a virus into a hereditary determinant of a cell Under the impact of the discoveries about lysogeny the focal interest of many phage workers as well as of students of other viruses has shifted from the purely destructive parasitic aspects of virus infection to its integrative aspects Lysogeny typically an instance of latent virus infection has become a branch of bacterial genetics The purpose here is to emphasize this genetic integrative aspect of virus infection and to outline our current work on the mechanisms by phage gives rise to prophage that is to an integrated constituent of th genom

* rted by the American Cancer Society Inc and from the University
Unive

Penetration of the genetic material of a temperate phage into a cell gives rise either to a lytic cycle, in which *vegetative phage* presumably DNA multiplies in a non infectious condition matures and is liberated by cell lysis or to a lysogenic cycle, in which the infected cell survives and the phage becomes *prophage* permanently established in at least some of the progeny of the surviving cell

The nature of the vegetative cycle has been studied extensively both at the chemical and at the genetic level (9 10 17 27 40) In addition to controlling the specificity of its own reproduction, the vegetative phage may bring about in the host cell destined for lysis a number of other functional alterations Of these some, like the biosynthesis of hydroxymethylcytosine deoxyribotide induced by the T even phages (10) or the restoration of thymine synthesis in thymine requiring mutant bacteria (1), fulfil requirements for the synthesis of phage itself I have pointed out elsewhere (31) that all these activities can be interpreted as due to genelike functions of the phage DNA both autocatalytic and heterocatalvtic in nature These genetic functions need not be exerted by the phage genome in physical and functional isolation Even while driving the host cell to lysis and death the vegetative phage may achieve some degree of physical integration with the host genome The suppression of host specific syntheses by certain phages (9) can be interpreted as a selective incapacitation of the host genome by an incompatible phage genome that is as genetic parasitism (29) Likewise the phenomena of host induced modification (30) and of reactivation between phage and host (13) have suggested some close interactions between vegetative phage and host genome Recent observations on the determination of bacterial antigens by phage during the lytic cycle to be discussed later in this paper reveal a basic similarity between the genelike functions of phage in the vegetative and in the prophage states

It is however in the prophage condition within lysogenic bacteria that the genetic features of phage are most clearly observed Just as the term gene' has assumed the dual meaning of a factor controlling hereditary traits and of a material chromosomal segment whose changes affect that trait thus prophage has developed the dual meaning of the hereditary ability to produce a specific phage and of the material element controlling this property The association of this material element with the host cell chromosome has been demonstrated in a variety of ways (21) The precise details of this association—whether linear within the linear chromosome or by sidewise pairing with a prophage homologous region of the chromosome or variously branched—are still unknown The chromosomal location of a given prophage may be unique as for lambda in *Escherichia coli* or multiple as with P2 (4) and S1 (11)

Just as a gene must be attributed both autocatalytic and heterocatalytic

functions, so a prophage controls other properties besides its own perpetuation and the potential ability of the cell to produce phage Some of these properties still pertain to phage infection prominent among them is the immunity of a lysogenic cell to lysis by superinfection with phages related to its prophage Other properties have no apparent relation to the functions related to phage production or immunity Here belong the control of toxin production in *Corynebacterium diphtheriae* (14), of certain somatic antigens in *Salmonella* (19 38), and of other properties which are regularly associated with the presence of specific prophages and disappear with loss of prophage Here belongs also 'prophage interference, that is, the interference by a prophage with the multiplication

TABLE 1

SOME PHAGE DETERMINED CELL PROPERTIES AND THEIR MODIFICATIONS BY VARIOUS FACTORS

PROPERTY	MODIFYING FACTOR		
	Phage Mutant	Host Genome	Environment
Phage production	Defective (20)	Reduced in streptomycin resistant mutants	Suppressed at low temperature (7)
Immunity to related phages	Dismune (5)†		
Prophage interference with growth of unrelated phage	Suppressed in some defective lysogenies	Suppressed by streptomycin resistance (25)	
Toxin production in *C diphtheriae*	Atoxic (15)	Absent in some host strains (36)	Dependent on medium composition (35)

D ta Pl f m u p ll h d m te l by J N Ad ms
† These ch ges may be ec mb nat nal rath tha mutat al (Z n J r B ta perso l c mmun cat n)

of other unrelated phages (25) In these instances we speak of ' conversion of cell properties by acquisition or loss of prophage

The point that I wish to emphasize is that these phage controlled properties behave exactly like any other hereditary cell properties with respect to their determination Like other properties they can be altered by genetic changes in the phage itself, and their expression is affected by changes in the genetic background of the host cell and by specific environmental factors For example toxin production in *C diphtheriae* can be abolished by recombination between a toxigenic and a non toxigenic phage by placing a toxigenic phage in a different host cell and as is well known by changing the growth medium Other examples are listed in Table 1

It is especially important to note that even those phage controlled cellular properties that concern more directly the phage itself behave in the same

fashion Thus immunity, which generally extends to all prophage related phages, can be restricted in dismune prophages (5) and is presumably an expression of a physiological, heterocatalytic function of the prophage rather than of steric hindrance to the multiplication of similar phages by an established prophage Even the production of mature phage from prophage may be suppressed either by environmental conditions such as low temperature (7) or by mutation to a defective prophage (20) In lambda a defective mutant prophage gives abortive maturation producing incomplete non infectious viral elements liberated by cell lysis whatever infectious phage is produced stems from reverse mutations to non defectiveness The site of a defective mutation can be mapped within the phage

Clearly, a defective prophage comes close to being an exogenous determinant of heredity without viral function Yet the defective lambda prophage still has a destructive effect on the cells in which it matures in fact mass lysis can still be provoked by maturation inducing agents such as ultraviolet light Recently however, a more extreme situation has been discovered in our laboratory by Dr Hisao Uetake In cultures of *Salmonella anatum* that have been lysogenized with phage ϵ^{15} occasional non lysogenic colonies are found The lysogenic cells are induced to lysis by ultraviolet light some of the non lysogenic derivatives are not Yet they are not altogether prophage free Prophage ϵ^{1} controls the conversion of *Salmonella* somatic antigens from 3 10 to 3 15 and cells with antigen 15 cannot adsorb phage ϵ^{1} The non lysogenic derivative still has antigen 15 and is unable to adsorb phage ϵ^{1} It has lost by mutation *both* the maturation property and the inducibility by ultraviolet light but retains the genetic determinant for antigen 15 Should the residual genetic element responsible for the antigen 15 be called a defective prophage? The antigenic character has become a *purely bacterial character* without any detectable tie to a phage transmissible determinant Thus through lysogenization followed by mutation we witness infectious heredity become non infectious

We are naturally led to ask, Is the infectiousness restricted to some genetic determinants? Or can any determinant possibly by mutation become infectious? The transduction phenomenon indicates that all bacterial heredity can be infectious by what is supposedly an accidental transmission within a virus vehicle (42) Whether any fragment of a bacterial chromosome may become a virus by acquiring the ability to control its own maturation into a specific transmissible element remains for future work to decide

THE ESTABLISHMENT OF LYSOGENY

Infectious heredity requires the integration of the exogenous controlling factor into the functional organization of the recipient cell Our recent work

has been directed at elucidating this process of integration in the establishment of the prophage Two aspects may be considered the material integration of the prophage into the cell structure and the biochemical integration of pro phage controlled functions into the network of cell functions It is with ap proaches to these two problems that I shall mainly concern myself here

Most of our work concerns phages of *Salmonella* In a cell infected with a *temperate phage there occurs an early decision as to whether lysis or survival* will ensue The decision can be influenced by a number of environmental factors (33) and by the genetic properties of the phage itself *Virulent* phage mutants fail to establish lysogeny In phage P22 of *Salmonella typhimurium,* we observe a number of such mutations to virulence (26) The remarkable fact is that different virulent mutants in mixed infection can co-operate to establish lysogeny We interpret this co-operation as evidence that the path to lysogeny includes a number of physiological steps Each step is subject to mutational blocks and any one block forces the choice into the path to vegeta tive growth and lysis Two mutants blocked at different steps can co operate with each other by physiological supplementation in producing lysogeny Thus there is a definite prospect of interpreting the phase of decision in terms of biochemical reactions Indeed, L E Bertani (8) has recently reported that treatment with agents that suppress protein synthesis such as chloramphenicol, can shift the outcome of infection from lysis to lysogeny

The infecting phage element itself must undergo rapid changes in state in these early phases R Ting in our laboratory finds that in a suitable medium, chloramphenicol added within a few minutes after infection can actually suppress phage infection altogether leaving a cured phage sensitive cell Which stage in phage development is subject to this "chemotherapy is still unknown but this observation indicates that protein synthesis is needed to protect the functional integrity of the initiating phage element within the newly infected cell

Once the choice toward lysogeny has been made how does the initiating phage give rise to prophage that is to the stable genetic determinant of ly sogeny? Usually the cells that survive infection give a progeny that is only partly lysogenic non lysogenic cells are segregated ' out for several generations (28) With phage P22 of *S typhimurium* L E Bertani and I infected cells with a mixture of genetically marked phage particles, let individual cells mul tiply in isolation for 6 to 8 generations to give clones of 50 to 250 cells and tested the cells of many clones for the presence and type of phage carried We observed extensive segregation of sensitive non-carrier cells in practically every clone Some carrier cells continued to carry more than one type of phage for many generations, even after colony formation and re streaking, but

ultimately they gave progeny stably lysogenic for one prophage only either a parental or a recombinant type Clearly, we were dealing with a complicated situation which could not be explained by assuming for example an early establishment of a prophage in one cell nucleus followed by nuclear segregation The unstable phase lasted too long and the carrier cells produced too great a variety of phage types

D K Fraser and I have undertaken to examine this problem by the detailed analysis of single clones of *S typhimurium* infected with phage P22 using micro manipulation by a modified De Fonbrunne technique (12) Each clone represents the progeny of a single cell that has been infected with phage particles of one or more genetic types The proportions and types of the infecting phages can be varied in such a way as to trace characters derived from a single infecting particle Thus for example when bacteria receive many particles of a virulent phage mutant and a single particle of a temperate one any infected cell that fails to lyse will be mixedly infected (26) and the clone to which it gives rise will reveal the fate of the genes derived from the minority parent phage

As each cell of a clone divides the daughter cells are separated from one another into droplets of nutrient medium No opportunity for reinfection is allowed in this process The separation is continued for 5 or 6 generations after which the separated cells are allowed to multiply in the droplets The cell progeny (or subclone) in each drop can then be sampled grown further and tested for the presence or absence of phage and for the type of phage it produces The absence of phage characterizes fully sensitive subclones the presence of phage in a subclone is evidence that the subclone derived from a cell that still contained at least one *functional* phage element Phage containing subclones that still throw off sensitive cells are readily detected If a cell lyses in a droplet before giving rise to a subclone the phage produced is withdrawn and tested

Figure 1 shows as an example the pedigree of a clone from a cell infected with a mixture of two temperate phage strains differing in two characters so that recombination can be observed

The results of this work are by no means complete but a few general patterns have emerged First the infecting particles are not distributed at random in functional form among the early progeny cells since early repeated segregations of sensitive cells can occur after infection with high phage multiplicity In some instances even with multiple infection a phage element can be maintained for several generations by single unilinear transmission within a clone that continues to produce non carrier sensitive cells as though the phage element did not multiply but continued to be handed over to one daughter cell at each division Such clones occasionally give a typical lysogenic subclone where each cell is a stable carrier

More frequently, a clone includes a variety of sublines, containing any one of the possible combinations of the infecting phages or of their recombinants, carried singly or jointly Stable lysogenic cells are formed by sporadic events

Thus we recognize an early phage carrier state, characterized by instability and segregation of non carrier cells during which extensive reproduction of phage elements must take place, enough to keep approximate pace with the repeated cell divisions Also, recombination must take place in this phase, since unstable sublines may carry only a recombinant type or, occasionally, a mixture of a parental and a recombinant type

Two remarkable features are, first, the not infrequent persistence of the unstable condition over scores of generations unstable sublines, which segregate sensitive cells and sometimes also stable lysogenics, can be found even after repeated single colony isolations Second multiple phage elements may persist in this unstable form (this did not occur in the pedigree of Fig 1) An unstable, multiple carrier subline may segregate out sensitive cells for several generations Other multiple carrier sublines which do not segregate out sensitive cells, segregate instead single lysogenics, all of which carry the same type of prophage

Our results taken as a whole, fit the following tentative picture In the early generations as well as in the unstable lines the progeny cells (or rather, presumably their nuclei) can carry a plurality of determinants whose multiplication is only roughly geared to a uniform pace Stable lysogenization consists of the permanent fixation of a phage element as prophage to a (presumably specific) location in the host genome The lines that segregate sensitive cells have this location still unoccupied The unstable phage elements may be either attached at other locations or less permanently fixed to the choice location or floating free within nucleus or cell The uniqueness of the stable type produced by segregation from the unstable multiple carriers would indicate that genetic recombination is less frequent between the supposedly established prophage and other phage elements within the same cell than between the unestablished elements in the early clone stages In the unstable lines that segregate sensitive cells prophage fixation is less frequent than in the early clone phases possibly the phage element is so located as to prevent stable incorporation

Lysis is never observed early within clones carrying temperate phages (the frequency of lysis is about 10^{-4} among lysogenic cells) Frequent lysis is observed however, in clones where a parental phage carried a gene for inability to become prophage Figure 2 shows the pedigree of a clone employing a mixture of two virulent phage mutants, c_2 and c_1, which can co operate to produce lysogeny (26) A phage with c_2 is unable to become a stable prophage, and the cells that carry it ultimately lose it or lyse hence all stable lysogenics in such a clone carry c_1 type phage When, however, early lysis occurs, the cells

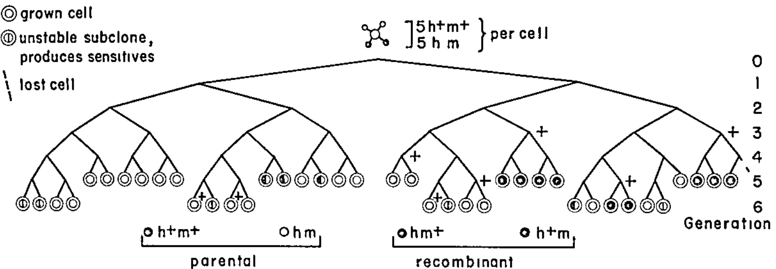


FIG 1 —Pedigree of a clone from a cell of a culture of *S typhimurium* infected with phages P22 *hm* and P22 h^+m^+ (multiplicity of infection 5 each) The blank circles represent cells giving rise to a pure sensitive subclone The colors in the other circles represent the genetic type of phage produced by the corresponding subclone The half colored circles indicate phage carrying subclones that still give sensitive progeny The + signs indicate the presumed events of establishment of a stable lysogenic subclone Note that no multiple-carrier cell was found in this clone

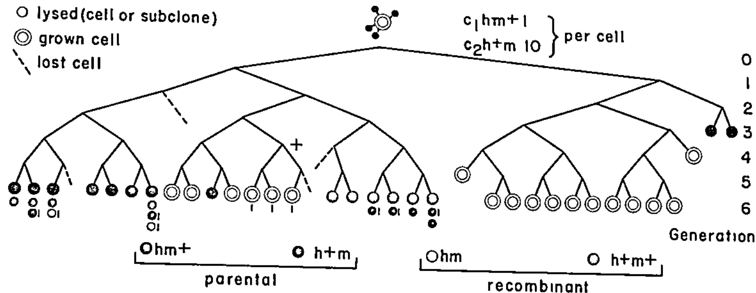


FIG 2.—Pedigree of a clone from a cell of *S typhi murium* (initial infection with phages P22 $c_1\ hm^+$ and P22 $c_2\ h^+m$ at multiplicities of 1 and 10 respectively) Symbols as in Fig 1 The circles without surrounding lines represent subclones that lysed completely either at the one-cell state or within a few generations The small circles represent minority phage types liberated by these subclones in addition to the majority type The sign *I* indicates phage of c_1 type all other phages were of c_2 type

produce *c* type phage either alone or as the majority type This might be due to an attempted attachment of the *c* type to the choice location which will result in immediate lysis (generally within one cell division) unless the cell continues to be protected by the presence of another non-c_2 phage element

Although this picture does not provide an insight into the mechanism of prophage fixation, it focuses our attention on the unstable phase of the process in which the genetic material of phage is propagated in incomplete synchrony with that of the cell In this state a phage carrier cell is like a heterogenote (34) which carries two or more homologous fragments of genetic material one of which is more stably integrated within the cell genome In fact it may be worth emphasizing that the unstable heterogenotic condition seems always to accompany infectious heredity whether it be lysogenization transduction transformation or mating The essence of all these proces es is the transfer of a genetic fragment from one cell to another In all these processes the exogenous fragments can apparently persist and multiply for a long time before becoming permanently incorporated into the cell genome The eventual incorporation may require specific pairing between the exogenous element and a homologous location followed by copy choice recombinational replication (23) and it may actually be prevented by an unsuitable location of the exogenous element with respect to the choice location

In lysogenization the unstably carried phage element resembles the exogenote of a heterogenote (34) rather than the pre prophage defined by G Bertani (2) as the non multiplying persisting form assumed by a phage when it superinfects an immune lysogenic cell Yet even in immune cells exogenous phage types may multiply to some extent (3) There is probably a continuous series of situations intermediate between non reproducing phage elements passively handed down from cell to cell and the established prophage In lysogenization we have observed all possibilities from the unilinear non replicating transmission as in abortive transduction (24) to the unstable single carrier state the multiple unstable carrier state and the established prophage state A clarification of the mechanisms of integration and of the physical and chemical state of the genetic elements in these various states can explain not only the process of genetic integration but also the organization of the bacterial chromosome and the limits of its accessibility to infectious variation which may play a major role in the evolution of bacteria and of other cells

THE INITIATION OF PHAGE CONTROLLED FUNCTIONS

My last topic concerns a system for the study of the functional activities of newly entered phage elements with regard to cell properties The system used has been developed by H Uetake It consists of *S anatum* (O antigens

3 10) and temperate phage ϵ^1, which, upon lysogenization converts the O antigens to 3 and 15 (38) The antigens 10 and 15 can be detected by agglutination with specific factor sera Uetake has isolated two other phages (both virulent mutants of temperate phages) unrelated to ϵ^1 Phage C_{341} adsorbs only to cells with antigen 10 phage ϵ^{34} *vir* only to cells with antigen 15 Since each cell infected by either phage is killed the system makes it possible to follow the phage receptors (and presumably the appearance and disappearance of the correlated O antigens) by following the kinetics of cell killing by the two phages in populations of recently infected cells

The results of these studies (39) are striking Agglutinability by the anti 15 serum appears in the infected population within 5–10 minutes after infection and reaches the full serum titer end point by 45 minutes Remarkably similar results are observed if the cells are infected *with a virulent mutant of* ϵ^1 which lyses all the cells with a latent period of around 60 minutes Clearly the control over antigen 15 is not restricted to the established prophage but takes place very soon after infection even in cells destined for early lysis

The study of sensitivity to the heterologous phages C_{341} and ϵ^{34} *vir* confirms this conclusion and extends it The cells surviving infection with ϵ^1 are largely resistant to phage C_{341} by 40–60 minutes after infection and are already killed by ϵ^{34} *vir* The slow rates of adsorption of these two phages in the transition period confirm a gradual appearance and disappearance of the cell receptors

Interesting results are observed by comparing the changes in phage sensitivity to the changes in carrier state in the segregation phase after infection with ϵ^1 Extensive segregation of non lysogenic cells takes place so that after 5–7 generations the initially infected population contains only about 10 per cent carrier cells a proportion which then becomes stabilized All non lysogenic cells give rise to colonies with antigen 10 all lysogenic cells give colonies with antigen 15 The phage receptors however lag behind Even the non carrier cells develop the new antigen and the new receptors to a maximal level before losing them again in later generations Reacquisition of sensitivity to C_{341} by the non carrier cells and loss of sensitivity to phage ϵ^{34} *vir* are not complete even after 10 generations In the meantime a significant proportion of the cells can adsorb both phages revealing a mosaic of the two receptors which like the correlated antigens are mutually exclusive in the stable states

In the transition stage the cell can apparently make an antigen and phage receptor whose genetic determinant has been segregated away The residual synthetic ability might then be due to a primary gene product possibly a non reproducing gene copy as may be the case in abortive transduction (23 24 37), or to a more distant gene product possibly enzymatic in nature

produced in excess under phage control and slowly diluted away after the controlling element is gone

This system presents definite advantages for the study of phage controlled properties It makes it possible to detect a series of apparently unrelated functions of an exogenous genetic element, including control of phage production cell antigens and possibly other properties such as immunity to lysis by reinfection with the carried phage The rapidity with which the new functions appear also suggests applications to biosynthetic studies, since it should be feasible to study the rapid formation of a gene product in cells that started with none at all Finally, a comparison between the functions of the exogenous genetic element in its various states—the vegetative the unstable prelysogenic and the established prophage—should provide information about the functional integration of the bacterial genome

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CHAPTER 14

The Nucleoprotamines Their Formation and Their Function

Kurt Felix

THE nucleoprotamines are salts of the basic protamines with desoxyribo nucleic acid They were first discovered in the nuclei of fish sperm The corresponding constituents of the nuclei in the somatic tissues are the nucleo histones Since the sperm nucleus contains most of the hereditary materials which are transferred from the fish father to his offspring the nucleoprotamines must have something to do with this function

Before I go into the details I should like to summarize how the nuclei of fish sperm are prepared We collected the fresh milt of several species of trout in a fish hatchery near Frankfurt Germany and suspended it in distilled water By this treatment the cytoplasm and tail were detached from the nucleus homogenization for only a short time separated them completely Finally the nuclei were centrifuged off and thoroughly washed with distilled water until the reaction for tryptophan was negative then we were sure that the cytoplasm had been completely removed

Some electronmicrophotographs of intact trout sperm and the different stages of cytolysis are shown in Figure 1 You will notice that the nuclei did not change in volume shape contour or optical density because of this treatment To investigate whether they preserved their biological capacity we injected them into the corresponding eggs (into each egg only one sperm nucleus) (1) This is a very difficult task beset with many casualties and frustrations First the nuclei are very small and have to be sucked into a thin capillary tube only 10 μ in diameter Sometimes one catches a dust particle instead When one punctures the egg to inject the nucleus some of the fluid pours out and may wash the nucleus out again We treated two to three hundred eggs of each spawning in this way A total of about seventy of them developed to normal fish Unfortunately since none lived longer than a year and a half we could not determine the sex

However I believe that our injected nuclei have induced true fertilization The only difference between our procedure and the natural process is that we injected the nucleus only whereas in nature the whole head including not only the nucleus but also a slight amount of cytoplasm which surrounds it, enters the egg I do not think that such a tiny quantity of cytoplasm is of great importance



Fig 1 —Electron micrographs of trout sperm (9350×) a In Ringer s solution fixed with osmic acid b in distilled water 10 minutes fixed with osmic acid c in distilled water 30 minutes fixed with osmic acid d naked nuclei

Others are opposed to this conclusion and consider our fish to be parthenogenetic To exclude this we sent two trout to Mrs Leuchtenberger one derived from a naturally and the other from an artificially fertilized egg Both were one year old She determined the relative DNA content in the nuclei of the

liver cells of these two trout but did not know which was which Her results are shown in Figure 2

There is no difference between the two sets of measurements Fish No 2 was the artificial one Both livers contained the same percentages of DNA corresponding to diploid tetraploid and octaploid nuclei If it is true that the DNA content corresponds to the number of chromosomes then both fish had the same number

We have additional evidence for true fertilization There are two species of trout *Salmo trutta* and *S fontinalis* the individuals of which can be crossed We performed this cross artificially by injecting the nucleus of *S fontinalis* into the egg of *S trutta* One egg out of a hundred developed normally

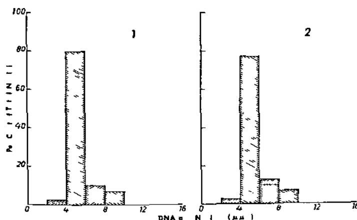


FIG 2 —Distribution of DNA determined by microspectrophotometry among individual nuclei in liver cells of two German trout *1* Fish that developed from naturally fertilized egg fish from egg artificially fertilized with naked sperm nucleus Each histogram based on 30 nuclei Mean DNA per nucleus 5 07 $\mu\mu g$ (Courtesy Mrs C Leuchtenberger)

I do not need to mention that hundreds of control experiments were performed in which we punctured the eggs or injected the suspension fluid only None of these eggs lived longer than 4 weeks

Therefore I want to repeat that I believe that our naked nuclei in spite of the treatment with distilled water preserve their biological capacity and consequently still contain all the information about the hereditary qualities that is transferred from the fish father to the offspring Accordingly we may feel justified in drawing conclusions from the chemical analysis of the isolated nuclei

The naked nuclei are insoluble in distilled water but completely soluble in 10 per cent sodium chloride When this solution is poured into distilled water all nuclear material is precipitated as a fibrous mass Nothing remains in solution The fibers have the same composition as the nuclei (Table 1) (2 3)

As you know the nucleoprotamines are salts of the basic protamines with desoxyribonucleic acid (DNA) There is no ribonucleic acid in the sperm nucleus The protamines are simple proteins with molecular weights varying between 4 000 and 6 000 They contain only six to eight amino acids among which the basic ones predominate The amino acid pattern varies from one species of fish to another, as Table 2 demonstrates (2, 4 5) Here you see the analyses of the protamines of seven fish species Alanine serine, and arginine occur in all of them also glycine since we have detected it recently in clupeine Valine and proline are missing only in sturine The other amino acids vary to a

TABLE 1

COMPARATIVE ANALYSES OF SPERM NUCLEI AND OF FIBERS DERIVED THEREFROM

	N	P	N/P	ARGININE	P/ARGININE
	Nuclei				
Salmo irideus	19 67	5 87	3 35	30 86	1 0 94
S trutta	19 80	5 72	3 46	30 42	1 0 95
S fontinalis	19 78	5 88	3 36	30 44	1 0 92
Clupea harengus	19 73	5 76	3 42	30 42	1 0 94
	Fibers				
Salmo irideus	19 52	5 65	3 43	30 60	1 0 96
S trutta	19 67	5 71	3 44	30 ⸤0	1 0 94
S fontinalis	19 67	5 73	3 43	30 15	1 0 94
Clupea harengus	19 57	5 68	3 44	30 77	1 0 96

TABLE 2

AMINO ACID COMPOSITION OF SEVEN PROTAMINES (MOLECULAR RATIOS)

	Iridine (*S irideus*)	Truttine (*S trutta*)	Fontinine (*S fontinalis*)	Lacustrine (*S lacustris*)	Salmine (*S salar*)	Clupeine (*Clupea harengus*)	Sturine (*Acipenser sturio*)
Glycine	2	2	2	2	3	+	2
Alanine	2	2	2	2	2	4	5
Serine	3	3	3	2	5	6	3
Threonine	0	0	0	0	0	2	1
Valine	4	5	5	2	4	4	0
Proline	5	5	5	5	5	5	0
Isoleucine	1	0	0	1	0	1	2
Aspartic acid	0	0	0	1	0	0	0
Glutamic acid	0	0	0	1	0	0	1
Arginine	500	50	50	40–50	55	53	35
Lysine	2	0	0	0	0	0	9
Histidine	0	0	0	5 15	0	0	7

greater extent As far as one can judge from the present analyses no sulfur containing amino acids and no tryptophan occur in the protamines I believe that these amino acid patterns are characteristic of the fish species The two kinds of trout *S trutta* and *S fontinalis* which can be crossed have protamines of the same amino acid composition It is perhaps for this reason that they can be crossed

The DNA has in contrast to the protamines almost the same composition in all the species we have investigated varying only to a very small extent Only in regard to methyl cytosine does there seem to be a significant variation (Table 3) (6)

In the nucleoprotamines the phosphoric acid residues of DNA are bound to the basic amino acids especially to arginine Therefore the ratio of phosphorus to arginine is almost exactly 1 except in the nucleoprotamine of the sturgeon

TABLE 3

DISTRIBUTION OF PURINES AND PYRIMIDINES IN DNA PREPARATIONS

(Moles per 100 Atoms of Phosphorus)

Source of DNA	B				
	Ad	Thym	G	Cytosine	Methyl-cytosine
Salmo irideus	28	26	21	20	+
S trutta	28	26	21	20	−
S fontinalis	27	26	20	20	+
S salar	27	26	21	21	−
Acipenser sturio	29	27	22	20	−

Sturine contains lysine and histidine in addition to arginine and some of the phosphoric acid residues are bound to these bases

Judging from the polypeptides isolated from partial clupeine hydrolyzates the arginine occurs almost entirely in sequences of four residues and these tetrapeptides of arginine alternate with dipeptides of monoamino acids The peptide chains of most of the protamines begin with proline at the nitrogen end (7–11) This is followed by another monoamino acid which is alanine in the case of clupeine Then comes the first tetrapeptide of arginine and in the further sequence dipeptides of monoamino acids alternate with tetrapeptides of arginine Finally the chains end with a dipeptide of arginine at least in the case of clupeine (12)

According to present views of the function of the nucleic acid in protein synthesis one would expect a regularity in sequence of the nucleotides similar to the arrangement of amino acids in the protamines But the composition of herring DNA does not allow us to construct such a regularity There is no

purine or pyrimidine base that occurs in a pattern corresponding to that of arginine in clupeine (6)

Some nuclei and the corresponding nucleoprotamines contain aspartic and glutamic acid which as yet we have not found in the corresponding isolated protamines This is especially the case in the nuclei and nucleoprotamines of the rainbow and European and Canadian brook trout We have not yet found out how these dicarboxylic amino acids are bound in the nucleoprotamine but we are sure that they are present

If it is true that the protamine is the protein of the generative cells then one has to inquire whether it occurs also in the sperm nuclei of higher animals Its presence in rooster sperm has been proved by Mirsky and his co-workers (13)

TABLE 4

MOLECULAR RATIOS OF AMINO ACIDS IN ROOSTER PROTAMINE

	Gallin (Mirsky)	Gallin HCl (Fischer Kreuzer)
Arginine	45	44
Histidine	2	Absent
Glycine	10	1
Serine	14	5
Threonine	3	2
Alanine	4	5
Valine	2	3
Proline	7	5
(Iso)leucine	1	1
Aspartic acid	1	Absent
Glutamic acid	2	1
Tyrosine	6	Absent

and their results have been confirmed by Fischer and Kreuzer (14) The latter authors have isolated the protamine in a purer state and have proved by the determination of the amino acids that it is a real protamine, as can be seen in Table 4

From the sperm of mammals no protamine has yet been isolated We have tried it several times with bull sperm but without success However, there are striking similarities in composition between the head of bull sperm and the nucleus of trout sperm The head of bull sperm contains 25 36 per cent of arginine whereas a nucleus of trout sperm contains 30 57 per cent The phosphorus content of a bull sperm head is 3 98 per cent and the corresponding figure for trout sperm is 5 82 per cent The molar ratio of arginine to phosphorus in bull sperm is 1 13 The slight excess of arginine is due to the presence of other proteins present besides the hypothetical protamine we found fifteen amino acids in the total protein and among them were cystine and tyrosine which

usually do not occur in protamines The nitrogen content of bull sperm head amounts to 19 76 per cent and the ratio N P is 4 96 compared with 3 4 for trout sperm Since a bull surely has more genes than a trout the composition of bull sperm must be more complicated than that of trout sperm

For discussion of the question as to how all the information about the hereditary qualities can be represented in a sperm nucleus let us go back to the trout because there the situation is much simpler At the beginning I pointed out that its nucleus consists of nucleoprotamine only All the phosphorus in it belongs to DNA and all the nitrogen belongs to nucleoprotamine including the above mentioned fraction which contains the two dicarboxylic amino acids If later another constituent should be found it would be one without nitrogen and phosphorus Once we suspected that a steroid might be present Dr Hubener in Frankfurt has carefully investigated the nuclei of the brook trout but could not detect any of the known steroids

Thus our question about information content is narrowed to the more specific one How can the nucleoprotamine represent the hereditary qualities According to our present knowledge and theories each quality is represented by one special gene I do not know how many genes a trout possesses let us assume a thousand just for easier calculation

According to our analyses a gene if it is a definite substance at all must be a nucleoprotamine in the case of a sperm nucleus Can the two components of the nucleus—the protamine and the DNA—form a thousand different nucleoprotamines? I am talking now about different nucleoprotamines within a single nucleus not about differences between fish species In this respect we have already learned from chemical analysis that the nucleoprotamines differ in the protamine part by the amino acid composition and in the DNA part by the content of methylcytosine Differences within a single nucleus are possible because the protamines and the DNA are not homogeneous substances but consist of several similar but not identical components

In our experiments on the homogeneity of the protamines we used chiefly clupeine the protamine of herring sperm because it is available in comparatively large quantities It can be separated into three fractions by paper chromatography by using a solvent mixture of 18 cc 86 per cent ethanol 6 cc 20 per cent trichloroacetic acid and 1 cc secondary butanol The separation depends on external factors we studied especially the influence of mineral salts These were added to the clupeine at the starting point of the chromatogram Out of twenty salts sodium oxalate was the most suitable the anions having greater influence than the cations In the presence of this salt the three spots were very distinct but these fractions did not differ in amino acid composition We obtained much better results with the countercurrent distribution of Craig using as lower phase a 15 per cent solution of sodium acetate in water and as

upper phase a 5 per cent solution of lauric acid in *n* butanol We worked with this system in our preliminary experiments three years ago and have tried in the meantime many other systems but none has been found more suitable than the first one

The clupeine methylester hydrochloride used for these experiments was prepared very carefully from fish milt which we collected in 1953 After the samples had been regularly distributed over four hundred units three distinct fractions were obtained The first was the slowest moving appeared to be almost homogenous and corresponds to a single component of the native clupeine The two others were still mixtures (Fig 3)

The analyses of the three fractions reveal clear differences in composition and in N terminal amino acids (Table 5) Fraction I consists of only five amino

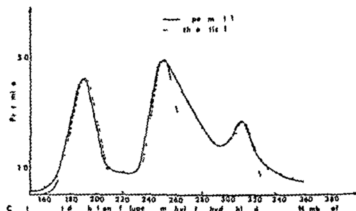


Fig 3 — Countercurrent distribution of protamine components of clupein

acids glycine threonine and isoleucine are missing The amino acid at the nitrogen end of the chain is proline Fraction II contains all amino acids of clupeine except valine and it is the only one of the three which contains glycine The different polypeptides making up this fraction all begin with proline Fraction III contains several at least three polypeptides One of them has serine as *N terminal amino acid and occurs in large amounts* whereas the two others ending with threonine and alanine, occur only in very slight amounts

Although the total number of polypeptide components in the native clupeine is not yet known for further calculation we shall assume that there are ten Even if there were only one kind of DNA we could now build up many nucleoprotamine molecules These molecules could differ according to which clupeine polypeptide components and how many molecules of each are combined with the DNA and further, according to their arrangement along the nucleic acid chain

In this herring nucleoprotamine the molecular weight of the DNA is approximately 1 000 000 and that of the nucleoprotamine 1 4 × 10[6] Taking 4 000 as an average molecular weight of the clupeine polypeptides then about a hundred individual polypeptides would be present in one molecule of nucleoprotamine If all ten kinds of polypeptide were represented by only one molecule each then 3 7 × 10[6] different arrangements of these polypeptides along the DNA chain would be possible The possible variability is even much greater because not all components have to be present their molecular ratio can vary widely and the mean number of polypeptides of each kind is ten instead of one

TABLE 5

AMINO ACID COMPOSITION OF CLUPEINE FRACTIONS OBTAINED BY COUNTERCURRENT DISTRIBUTION

Amino Acid	Fraction		
	I	II	III
Glycine	Absent	1	Absent
Alanine	3	2	2
Serine	2	3	3
Threonine	Absent	2	1
Valine	2	Absent	2
Proline	2	2	4
Isoleucine	Absent	1	Absent
Arginine	25	25	25
N terminal amino acid	Proline	Proline	Serine threonine and alanine in slight amounts
Minimal molecular weight	3 970	4 890	1 [illegible]20–5 160

The real possibilities for variation are still greater because the DNA also consists of several components Thus the same consideration can be applied to it

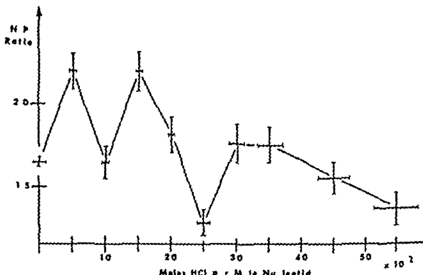
Dr R K Zahn has prepared DNA of herring sperm in a state of high polymerization Its composition is given in the accompanying table This DNA be

N	15 34%	Adenine	27 9%
P	9 3%	Thym	27 1
N P	1 65	Guanine	2[illegible] 2%
Bases	36 91	Cytosine	20 3%
		5-Methylcytosine	1 9%
		Uracil	0 6%

longs to the adenine thymine type The molecular ratio of the purine to the pyrimidine bases is 48 47 = 1 02 If the molecular weight of this DNA is assumed to be 1 000 000 then it would contain from 2 800 to 3 000 nucleotides There exists accordingly a very great number of possible permutations of their

arrangement in the nucleic acid chain even if the relative abundances of the four bases are the same for every DNA molecule

The opportunities for variability are further increased somewhat as with the protamines, because of the existence of several DNA components which vary widely in relative abundance of the bases Dr Zahn removed the electrolytes



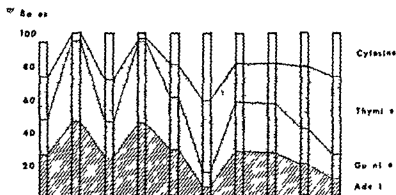


Fig 4—Base content of 10 DNA components separated by differential precipitation *Upper* relative amounts of total bases in the 10 fractions *Lower* relative amounts of the different bases in each of the 10 fractions

from his DNA by the use of ion exchange resins By this treatment the DNA becomes soluble in alcohol *If one adds increasing amounts of hydrochloric acid* or of a salt to a solution of electrolyte free DNA in water, several fractions of DNA are precipitated They differ not only in their total base content (Fig 4 *upper*) but also in the relative amounts of the four bases (Fig 4 *lower*) Three

fractions are especially remarkable two of them contain almost exclusively purine bases and the other one pyrimidine bases

The foregoing are the possibilities which according to the present state of knowledge enable nature to build up a great variety of nucleoprotamines so that each gene can be represented by a special nucleoprotamine But the large number of possible variations is probably restricted in practice by some ruling law

There remains a question concerning the number of nucleoprotamine molecules in the sperm nucleus Their number can be calculated from the phosphorus and nitrogen content of a single nucleus and from the molecular weights of DNA and nucleoprotamines The determinations have been recently repeated by Dr A Goppold Krekels and Dr R K Zahn in the Frankfurt Institute They have found in a single sperm nucleus an average of $2\ 16 \times 10^{-9}$ mg DNA for the brook trout and $2\ 6 \times 10^{-9}$ for the rainbow trout Taking $2\ 3 \times 10^{-9}$ mg as the mean value and assuming a molecular weight of 10^6 the number of DNA molecules per nucleus amounts to about $1\ 4 \times 10^6$ The number of nucleoprotamine molecules is of course the same

These molecules must lie very close together within the nucleus not randomly distributed but in a regular and definite order According to an X ray diagram taken by Wilkins and Randall (15) the bundles of the nucleoprotamine fibers are arranged in a three dimensional order Unfortunately we do not yet have in Frankfurt the facilities to make thin sections through the sperm nuclei A recent paper of G Yasuzumi (16) in Japan includes beautiful electron microphotographs of sections of sperm heads in sparrow testes in which the bundles of fibrils can be recognized Dr Hans Ris has been so kind as to furnish four electron microphotographs (Fig 5) for reproduction in this article C M S Dass made them in his laboratory They represent different stages of the developing testis of the grasshopper First (*a*) there are fibrils of 100 A thickness in the nuclei which are split into thinner ones of 50 A in some places Dr Ris supposes that these thinner fibrils correspond to the nucleoprotamines Lat[illegible] the fibrils form membranes (*b*) which are drawn closer together in the late[illegible] stages (*c d*) until they form a very compact almost homogeneous mass

Perhaps the three dimensional arrangement of these bundles also repr[illegible] some hereditary qualities Thus all chemical and physical properties of [illegible] nucleoprotamines may contribute to their function in heredity

Finally I want to mention that these nuclei contain no enzymes an[illegible] no oxygen No reactions are taking place in them they are [illegible]

During the last two years we have tried to find out how and in [illegible] sperm ripening the nucleoprotamines are formed Kossel (18) [illegible] question about forty years ago His opinion was that they are f[illegible] plicated proteins by slow and gradual transformation ultimat[illegible] the amino

acids that do not occur in the protamines are supposed to be metabolized until only those remain which are necessary for the transmission of the heredity and for the binding of the nucleic acid. Histones were supposed to occur as intermediates in this transformation.

To answer this question Dr. Goppold-Krekels collected the testes of Canadian brook trout (*S. fontinalis*) in nine different stages of maturity beginning with the ninetieth day before spawning. The intervals between the single stages

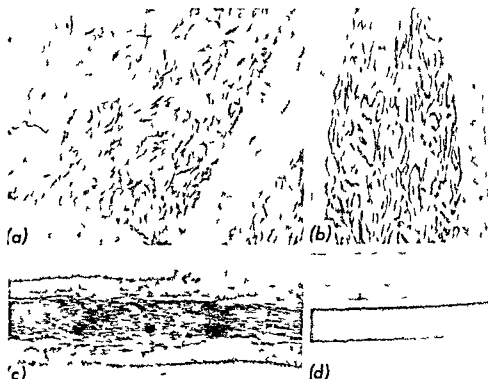


FIG. 5.—Electron micrographs (43 000×) of sections of grasshopper spermatozoa. *a. Romalea microptera*, beginning of elongation of nucleus. The fibrils are chiefly oriented in the direction of the nuclear axis. In some places they are 100 Å thick but show a splitting into finer fibrils about 50 Å thick. We think these represent the nucleoprotamines. *b. Chortophaga viridifasciata*. After the splitting into 50 Å fibrils these lie together in membranes that form reticular anastomoses. *c. Chorthippus*. With elongation of the nucleus the membranes are drawn out, at first in the form of a honeycomb, then the interstitial spaces become smaller and smaller, and finally the nucleus forms a uniform amorphous mass. *d. Chortophaga viridifasciata*. Nucleus of ripe spermatozoon. (Courtesy H. Ris.)

were about 10 days. At the beginning the testes were short and very thin threads. As time went on they swelled and finally became long thick sacs filled with spermatozoa.

The collected testes of each stage were homogenized in acetone and the gross particles removed by sedimentation. The acetone supernatant was filtered through gauze and centrifuged off. The sediment consisted almost exclusively of

nuclei as proved by phase contrast microscopy and was dried on filter paper To detect the first appearance of nucleoprotamine we could try to isolate it as a whole or only the protamine part The entire nucleoprotamine can be extracted by a 10 per cent solution of sodium chloride and precipitated by pouring the extract into distilled water as mentioned before The precipitate can be identified as nucleoprotamine by determining the amino acids by paper chromatography after hydrolysis The protamine part can be extracted separately with dilute (0 2 per cent) hydrochloric acid By this second method we expected to find some possible precursors of the protamine with basic characteristics

We used the NaCl method for investigation of all nine stages of the ripening testes The nucleoprotamine was present from the sixth stage on that is to say it was present 40 days before spawning and thereafter Before this time none could be detected by chemical means and from its first appearance the nucleoprotamine had the same composition as it had in the mature sperm We repeated the amino acid analysis of the nucleoprotamines in the four last stages of maturation and found no differences Furthermore we were not able to isolate any nucleoprotein in the sodium chloride extract which could be regarded as an intermediate step between the complicated precursor protein and the protamine Therefore we no longer believe that the protamines are formed gradually from complicated proteins by passing through intermediate stages They seem rather to originate by a special process during sperm ripening This does not exclude the possibility that the precursor is a complicated protein or a histone But this is transformed into protamine at once and not through intermediate stages as trypsinogen and chymotrypsinogen are transformed into the active enzymes by one single reaction (Perhaps the proteo hormones of the hypophysis are formed likewise from complicated proteins by proteolytic enzymes which occur in a rather high concentration in the gland)

When we extracted the nuclei with the hydrochloric acid the results were different We found a second basic protein in about relatively the same amounts through all stages which contains more amino acids than the protamine but could not be separated from it However Dr Goppold Krekels succeeded in separating the corresponding nucleoprotein It could be extracted with water from the dried nuclei and precipitated by trichloroacetic acid The precipitate contained the second basic protein combined with DNA From the water insoluble residue nucleoprotamine could be extracted very easily with 10 per cent sodium chloride (One can also extract first the nucleoprotamine and second the nucleohistone because the latter is not soluble in the salt solution) The second basic protein itself was obtained by extraction of the second nucleoprotein with 0 2 per cent hydrochloric acid and precipitation with acetone It had the qualities of a real histone It contained the three basic amino acids of the other amino acids we identified glycine alanine serine cystine threonine

aspartic acid glutamic acid methionine, valine proline (iso)leucine, phenyl alanine and tyrosine Tryptophan could not be found Since we found the nucleohistone in all stages even in the most mature one, just before the discharge of the semen we believe that it has nothing to do with spermatogenesis but originates from other cells of the testes

During our work we got knowledge of a paper that Max Alfert (19) recently published He investigated histochemically the problem of whether the nucleoprotein changes its composition gradually and progressively throughout all stages of meiosis or whether this change takes place suddenly in a particular cell generation He used as a criterion the absence of fast green staining after extraction of the sperms with hot trichloroacetic acid The protamines are soluble in the acid histones and other proteins are not With his technique he came to the same conclusion that the change from a histone type nuclear protein to a protamine occurs rather abruptly at a late stage of spermiogenesis This conclusion agrees with ours and we could confirm his results by chemical means

May I say a few words on the significance of the simultaneous occurrence of protamine and histone in the later stages of the ripening in the testes Histone is also present in the immature testes before the protamine appears It has also been identified by Mirsky and his co workers (20) in the erythrocytes of salmon and seems to occur in every somatic nucleus I think we may conclude that the histone is the genetic protein of the somatic cells and that consequently the protamines have to be considered as the genetic proteins of the generative cells Probably the mother cells of the sperms are equivalent to somatic cells containing a histone in their nuclei Therefore it may be that histones are the precursors of the protamines and are transformed into them during the ripening period by some proteolytic process or perhaps the protamines are produced somewhere else in the cell and replace the histones in the nuclei According to the zoologists the nucleus of the egg cell has the same composition as that of the sperm and also contains nucleoprotamine But it has not yet been analyzed chemically because it is very difficult to isolate it in a pure state The protamines of the combined nuclei have to be transformed into histones the genetic proteins of the somatic cells during development Perhaps this happens in that stage in which the cell nuclei of the gastrula lose their ability to induce the development of an enucleated egg cell to a complete frog according to the beautiful experiments of Briggs (21)

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CHAPTER 15

Mechanism of Viral Invasion*

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INTRODUCTION

DETAILED chemical information on the mechanism of viral invasion has been obtained only about the infection of bacterial cells by bacteriophage particles (1-5) Although a number of properties of the interaction between other viruses and their host cells particularly influenza virus and tobacco invasion virus have been described, it is not yet possible to formulate the chemical reac-

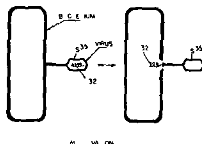


FIG 1 —Diagrammatic representation of the injection of viral DNA (containing P^{32}) into the host cell based on the experiments of Hershey and Chase (6)

tions which make up the infectious process in these systems In 1951 largely as the result of the experiments of Hershey and Chase (6), the invasion of *Escherichia coli* by bacteriophage T was given the descriptive term injection After adsorbing, tail first to the cell, the virus particle was thought to act as a ' microsyringe injecting only its nucleic acid into the host cell Figure 1 is a diagrammatic representation showing entrance of the P^{3} labeled deoxyribo nucleic acid (DNA P^{3}) into the cell while the great bulk of the S^{3} labeled viral protein remained outside the cell It was pointed out (6) that the nucleic acid and protein portions have separate functions the DNA is the carrier of the

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† Studies carried out during the tenure of a Lederle Medical Faculty Award

genetic information while the protein portion of the virus has the physiological role of insuring the introduction of the DNA into the host cell The investigations on the mechanisms of viral invasion have been largely concerned with the role of the viral protein

Even the limited information available in 1952 about the chemical nature of the host cell wall and the structure of the virus particle suggested that at least four separate steps occurred during invasion (1) adsorption of the virus particle tail first, to the host cell (2) alteration of the virus tail protein so that the nucleic acid could later be released from its protective protein covering (3) alteration of the host cell wall to permit the entrance of the nucleic acid and (4) the release of the nucleic acid from the head protein of the virus particle and its passage through the tail and into the host cell

VIRAL ADSORPTION

The initial step in the interaction of the bacteriophage with their host *E coli* B is considered to be the formation of reversible electrostatic bonds between sites on the virus particle and a complementary set of charges on the host cell surface (7) Specifically for bacteriophage T_2 positively charged amino groups on the distal end of the virus tail are attracted to negatively charged carboxyl groups on the host cell surface (8) That T_2 (and T_4) do adsorb tail first to the host cell was shown by means of electron microscopy by Anderson (9) and by Kellenberger and Arber (10) One further feature of the adsorption process which deserves comment is that for some virus particles such as T_4 adsorption occurs only in the presence of low concentrations of a co factor which has been identified as L tryptophan (11)

ALTERATION OF THE VIRUS TAIL STRUCTURE

In 1955 Kellenberger and Arber (10) presented electron micrographs showing that typical morphological changes occurred in the virus tail upon interaction with the cell wall Similar changes in the virus tail structure during phage–cell wall interaction were also later found by Brown and Kozloff (2) After interaction the tail was half its original length and was about 30 per cent thicker From a large portion of these altered virus particles a spikelike structure protruded from the end of the shortened and thickened tail Apparently the tail of these virus particles is composed of a number of structural components each of which it must be assumed plays a role in the infectious process

These observations raised two problems—(1) what chemical bonds were broken to cause these changes in the tail protein of the virus and (2) how these bonds were broken during the invasion process Practically simultaneously and quite independently various chemical and physical treatments were discovered which caused similar alterations in the virus tail structure (10 12 13) The only

chemical alteration which reflected any of the features of host cell–virus interaction was that produced by the action of complexes of the zinc group metals on T (12) After a detailed study of the action of these complexes on the virus tail protein and of the properties of these altered virus particles it was concluded that these metal complexes essentially duplicate the action of the cell wall on the virus particle (3, 4) The pertinent experiments which support this conclusion will be presented in this report

It was found that although $M/100\ Zn(NO_3)$ does not affect T the addition of various chelating compounds to the test system containing T Zn^{++}, and pH 7 5 tris buffer resulted in inactivation of the phage (12) Cyanide ions proved to be the most effective chelating agents Cyanide complexes of the other two members of the zinc group metals Cd^{++} and Hg^{++} were as active as zinc complexes in causing the inactivation of T_2 This type of inactivation of T was limited to the zinc group metals since cyanide complexes of Mn^{++} Ni^{++}, Co^{++} Fe^{++} Fe^{+++}, Cu^{+}, and Cu^{++} did not affect T phage

Four species of complexes—i e $Cd(CN)^{+}$, $Cd(CN)$, $Cd(CN)_3^{-}$ and $Cd(CN)_4^{-}$—are formed when NaCN is added to a solution containing $Cd(NO_3)_2$ The action of these four different complexes on Tr+ was studied and it appeared that the $Cd(CN)_3^{-}$ and possibly the $Cd(CN)_2$ complexes inactivate T while $Cd(CN)^{+}$ and $Cd(CN)_4^{-}$ appear to have little or no activity (3)

Preparations of T_2r^{+} treated with cadmium cyanide not only lose their ability to reproduce (i e they do not form plaques in the assay system) but also do not kill the host cells or attach to them in the normal manner When preparations of S^{3} labeled T_2r^{+} were treated with $Cd(CN)_3^{-}$ washed and then mixed with large amounts of host cells very little S^{3} sedimented with the host cells These results suggested that the virus tail which is the organ for attachment and for killing the host cell has been altered by the cadmium cyanide complex Electron micrographs of T_2r^{+} phage inactivated by cadmium cyanide (or zinc cyanide) showed that the distal half of the virus tail was removed in the reaction (12) Figure 2 shows four Tr+ particles which have been treated with $Cd(CN)_3^{-}$ and which illustrate the morphological changes in the virus in more detail The virus particles have heads which appear intact, but most of the distal tail structure has been removed The great majority of the particles have tails which are 50 ± 5 per cent (average of 60 measurements) of the normal length and about 30 per cent thicker than untreated phage particles In a small fraction of the altered particles (about 10–15 per cent) a spike protrudes from the center of the altered tail (see phage particle in the upper right hand corner of Fig 2)

The effect of cadmium cyanide complexes on the various T bacteriophages at 25° is shown in Figure 3 Only T phage was inactivated It should be noted that $T_2r^{+}h$ (the T_2 mutant which can invade host cell strain B/2) was inactivated much more slowly than $T_2r^{+}h^{+}$ and $T\,r\,h^{+}$ (the T mutants which cannot invade

B/2), which were inactivated at almost the same rate It is apparent that the change in the h locus affected the sensitivity of phage to inactivation by these metal complexes, while the change in the r locus did not affect the rate at which the virus was inactivated As the genetic evidence indicates that a change in the h locus affects only the structure of the protein at the distal end of the virus tail it may be concluded that the distal end of the virus tail is the site of action of the metal complex

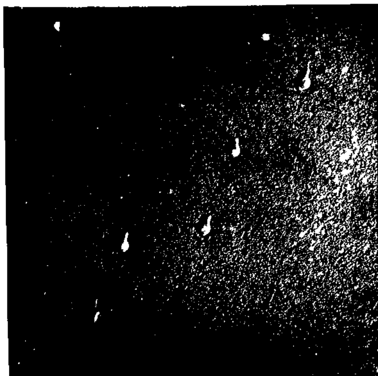


FIG 2—Electron micrograph of $T\ r^+$ treated with cadmium cyanide complexe for 1 hour at 37 pH 7 0 The sample was then diluted in water sprayed dried and shadowed with chromium 39 000×

It was found that at higher temperatures $Cd(CN)_4^-$ would inactivate both T_4 and T_6 the two phages which are closely related to T_2 After one hour at 37 T was 99 8 per cent inactivated T_4 was 93 per cent inactivated (and only in the presence of L tryptophan) and T_6 was only 35 per cent inactivated At 44 T_4 phage was slowly inactivated by $Cd(CN)_3^-$ even in the absence of L tryptophan but was very rapidly inactivated in the presence of L tryptophan (Fig 4) D Tryptophan could not be substituted for L tryptophan at either 37° or 44° Since L tryptophan is a specific co factor for T_4 adsorption to the host

cell, the rapid inactivation of T_4 in the presence of L tryptophan is additional evidence that the attack of the metal complex is on the distal end of the virus tail

In a study of the antigenic properties of the material released from the virus particle by the metal complex, it was found that $Cd(CN)_3^-$ removes material from the virus tail which still retains its unique and relatively large structure and can still react with anti T serum Certain bonds can be eliminated as pos

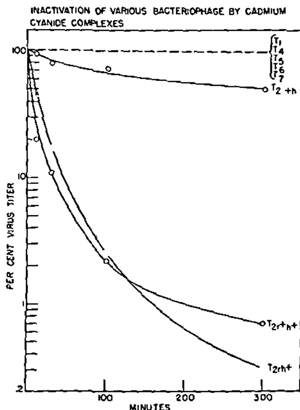


FIG 3 —Effect of cadmium cyanide complexes on various bacteriophages at 25 0 0063 M $Cd(CN)_3^-$ pH 7 0 ionic strength = 0 21 The solution containing the T had a final concentration of 0 025 mg ml of L tryptophan

sible links between the protein which is split off and the rest of the viral structure The metal complexes do not break ordinary peptide bonds oxygen ether or ester bonds or phosphate ester bonds Although our present knowledge of the reaction of zinc with proteins is slight, the most frequently considered binding site is to a sulfur atom (14) In this connection it should be noted that it has been found that the viral protein removed by the action of the metal complex contains about eight times the concentration of cysteine sulfur found in the rest of the viral protein (3) The direct identification of a bond involving the sulfur is

made difficult by its slow concentration relative to the rest of the viral protein. One T phage has approximately 3,500 cysteine residues (15) and only 8 per cent of these could be involved in the reaction with the metal complexes. This means that a change in three hundred bonds must be measured chemically on a structure containing about a million bonds (particle weight is approximately two hundred million).

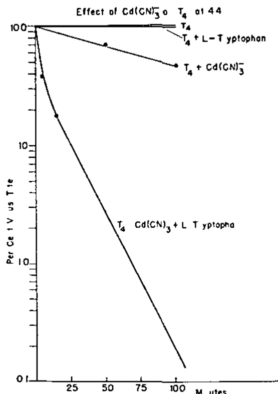


FIG 4.—Effect of cadmium cyanide complexes on T_4 r+ phage at 44. L-Tryptophan was added to a final concentration of 0.075 mg/ml. pH = 7.7. 0.0062 *M* $Cd(CN)_3^-$. Ionic strength = 0.21.

However, since sulfur bonds are known to link polypeptide chains, this possibility was examined, and the evidence to be presented is consistent with the idea that thiolester rather than the usual disulfide bonds are the main structural links in the viral tail. This evidence rests largely on the correlation of changes in the virus tail structure, usually as observed by means of electron micrographs, with the effect of various chemical and physical treatments. A list of various treatments which are considered to alter the tail structure of T_2 and T_4 and the effect of these treatments on thiolester and disulfide bonds is given in Table 1. It was found that the conditions affecting the rate of phage inactiva

tion by $Cd(CN)_4^{=}$ are in accord with the concept that the metal complex was catalyzing the alkaline hydrolysis of a thiolester bond in the viral tail protein (3) However in experiments with model compounds the metal complexes broke both thiolester and disulfide bonds (3)

Recently Kellenberger and Arber studied the effect of peroxide on T_2 and T_4 bacteriophage (18) They showed that incubating the virus particles for 10 minutes at 37° in 3 per cent peroxide in 10 per cent ethanol caused the characteristic alteration in the tail structure, the tail was shorter and thicker and had a spike protruding from the shortened end The results with peroxide alone would not differentiate between disulfide and thiolester bonds as the bond which is broken but together with the experiments described below, they support the conclusion that the structure of the virus tail is maintained by thiolester bonds

TABLE 1

TREATMENTS WHICH ALTER T_2 AND T_4

Treatment	Type of Bonds Broken	
	Thiolester $R-S-\overset{O}{\overset{\parallel}{C}}-R$	Disulfide $R-S-S-R$
1 $Cd(CN)_4^{=}$	+	+
2 H_2O_2	+	+
3 Thioglycollate	+	+
4 Papain	+	0
5 Heat	+	0
6 Freezing	?	?
7 NH_2OH	+	0
8 $(NH_4)_2SO_4$	+	0
9 (OH^-) pH 9 5	+	0

The effect of incubating $T_2 r^+$ phage with 1 *M* sodium thioglycollate for 3 hours at 37° is shown in Figure 5 The phage titer decreased about 70 per cent Various morphological changes are apparent in the six particles shown Particle *A* represents normal unaltered phage Particle *B* has a normal head but the distal portion of the phage tail protein has been removed and the particle is similar to the short thick tailed particles resulting after treatment with cadmium cyanide complexes Particle *C* has a normal appearing head but strands of material can be seen at the distal portion of the tail Particle *D* has the normal thin tail but the head is flattened and apparently empty of nucleic acid Particle *E* has a slightly flattened head, and again strands of material can be seen at the very tip of the almost normal tail Particle *F* has a badly flattened and nearly invisible empty head while all that remains of the tail is the quite resistant shortened and thickened proximal portion The fact that thioglycollate

causes these alterations of the viral proteins points strongly to a bond involving sulfur since the thioglycollate does not effect peptide ester or ether bonds but can undergo exchange reactions with thiolester or disulfide bonds

Papain has been shown recently to possess a powerful thiolesterase activity (16) which can be readily distinguished from its normal proteolytic esterase or



Fig 5 —Electron micrograph of T r phage treated with 1 *M* sodium thioglycollate The pH of the thioglycollate was adjusted to 7 0 before mixing with the phage The sample was incubated for 3 hours at 37 and diluted with 3 volumes of water sprayed dried and shadowed with chromium 61 500×

amidase activities The thiolesterase activity of papain is manifested in tris buffer without the need for either an activator such as cyanide or a metal chelating agent such as EDTA both of which are required for the other activities *Further citrate stimulates thiolesterase activity but not proteolytic activ*

ity The action of papain on T_2 and T_4 under various conditions is shown in Table 2 T_2 and T_4 were inactivated under conditions for thiolesterase activity, no cyanide or EDTA was needed and the inactivation was stimulated by citrate The relatively small inactivation of the T_4 by papain was found in four separate experiments The reduced susceptibility of T_4 to papain inactivation, as compared to T_2, parallels the relative susceptibility of the two viruses to $Cd(CN)_3^-$ inactivation In the absence of L tryptophan, T_4 was not inactivated

The morphological changes that papain produces in T_2 phage are clearly shown in Figure 6 In this experiment 50 per cent of the T_2 was inactivated, and normal phage with intact tails (particle *A*, for example) and phage with altered tails (particles *B* and *C*) are present in the expected ratios The action of papain on the virus tail caused the typical alteration noted before The phage tail was

TABLE 2

INACTIVATION OF T_2r^+ AND T_4r^+ BY PAPAIN

System	T_2 Inactivated 7 Hours at 37° (Per Cent)	T_4 Inactivated 12 Hours at 1 (Per Cent)
Complete	81	27
Complete minus EDTA	82	24
Complete minus citrate	67	20
Complete minus cyanide	88	27
Complete minus L tryptophan		0
Complete minus papain	0	0
Complete plus boiled papain	0	0

In m l t y t m th pH w 7 5 a d th e [illegible] ese 0 003 M t 0 02 M t t 0 0 M HCN 0 002 M EDTA 0 01 M NaCl d 2 mg ml of y t l l papa (N t t 1 R h m l) d th a e f T 0 04 mg ml f L-t ypt pha l tial ph ge t t n th y tem w s b t 5 × 10 pa t les/ml

shortened and thickened and from many of these shortened tails a small spike protruded

Gentle heating (17), as well as freezing and thawing (13) causes the typical alteration of the virus tail Thiolester bonds but not disulfide bonds are heat labile at neutral pH's Freezing and thawing treatment, which involves the formation of ice crystals is more difficult to evaluate when dealing with particles the size of a bacteriophage

Hydroxylamine has been shown rapidly to disrupt thiolester bonds (18) The rates of inactivation of T and T_4 by hydroxylamine are shown in Figure 7 The rate of T_4 inactivation was increased over six times in the presence of L tryptophan Although electron micrographs of hydroxylamine treated T_2 and T_4 have not been made, the effect of tryptophan indicates that the major attack on T_4 phage (and, by analogy on T phage) is on the tip of the tail Since

hydroxylamine has relatively little effect on disulfide bonds this experiment supports the concept that the virus tail contains thiolester bonds

The effect of two other reagents—weak base and high concentrations of NH_4^+—which also rupture thiolester bonds but not disulfide bonds was investigated The rate of inactivation of T_4 bacteriophage in 0 2 *M* tris buffer pH 9 5 or in 3 *M* $(NH_4)_2SO_4$ was measured in the presence and absence of L tryptophan

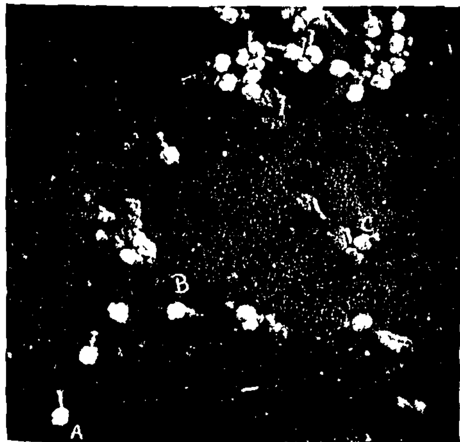


FIG 6 —Electron micrograph of T_4r^+ phage treated with papain 0 1 ml T_4r^+ suspension (4 × 10 phage particles) was incubated for 7 hours at 37 with 0 2 ml papain (0 5 mg/ml of 2× crystallized papain from Nutritional Biochemicals Inc) 0 05 ml 0 3 *M* tris buffer pH 7 5 and 0 2 ml 0 15 *M* NaCl The sample was then diluted with 4 volumes of water dried and shadowed with chromium 46 000×

These two reagents rapidly inactivated T_4 (more than 50 per cent in 20 minutes) and the rate of inactivation was increased about three times in the presence of 0 1 mg/ml of L tryptophan Here again although no electron micrograph is available, it is likely that the primary attack of these reagents is on the distal end of the virus tail

From these experiments it appears that the protein coat of T_2 bacteriophage contains four different proteins (1) head protein, (2) proximal tail protein (3) tail spike protein, and (4) distal tail protein The chemical nature of the tail spike is not known for certain, but it is not affected by DNAase and may be presumed to be protein More information is available about the protein at the distal end of the tail than for any of the other proteins When frozen and thawed (13) or treated with H_2O_2 (10) or with thioglycollate, four or five strands can be

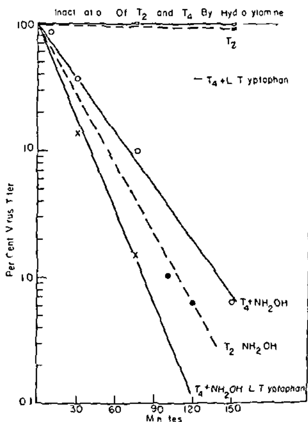


Fig 7—Inactivation of T_2 and T_4 bacteriophage by 0.05 M hydroxylamine pH 5.7 Incubation temperature was 37° L Tryptophan was 0.1 mg/ml

seen coming from the tail tip Presumably in the intact phage particle these strands are coiled about the center spike to give the characteristic bulge noted many years ago on the tail tip When these strands are removed by the action of $Cd(CN)_4^-$ they are still intact as judged by their antigenic properties Williams and Fraser (13) believe that these strands are the organs for attaching the phage to the host cell wall The evidence presented in this paper suggests that these strands are bound to the center spike (and possibly to each other) by thiolester bonds The structure of T_2 phage is illustrated diagrammatically in Figure 8

The relationship between these protein components of the virus particle and their role in the infectious process can be considered only after a discussion of other reactions which occur during invasion

THE ALTERATION OF THE HOST CELL WALL

Since the cell wall of *E coli*, strain B the host cell for the T series of bacteriophages is a rigid and complex structure (19 20) an alteration in the cell wall is required for the efficient injection of viral nucleic acid into the cell In 1951 Weidel (19) devised a method for isolating cell walls from *E coli* and re

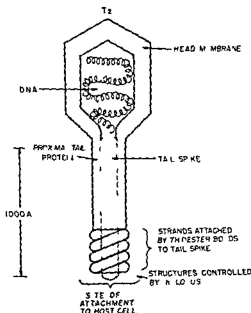


FIG 8 —Diagrammatic representation of the structure of bacteriophage T_2

ported that when these isolated cell walls were incubated with bacteriophage the cell walls were decomposed This was the first evidence that one of the steps of viral infection involved the alteration of the host cell surface The use of isotopically labeled cell walls appeared to offer the best means for studying the alteration of the host cell wall In 1954 Barrington and Kozloff (1) found that there was an enzyme in the tail of the *E coli* bacteriophages which caused the release of nitrogenous material from the bacterial cell wall (Table 3) However the exact nature of the alteration caused by the virus has not yet been determined

In view of the newer evidence on the complex morphology of the virus tail the localization of the enzymatic activity to the tail is not adequate and a study

of the morphologic localization of the enzyme was undertaken by Brown and Kozloff in 1957 (2) Figure 9 is a schematic diagram which illustrates the various morphologic forms of the virus particle which were tested for enzymatic activity on C^{14} labeled host cell walls

Whole T and T_4 bacteriophages liberated about 5 per cent of the total cell wall carbon in a non sedimentable form Short tailed T_2 and T_4 preparations

TABLE 3

INTERACTION OF CELL WALLS WITH T PHAGES

Phag	Phag Ad orbed (P r C t)	W ll N Made Non sed m nt bl (P r Ce t)	Wall N Made N n sed m nt bl p Ph ge (Mg ×10)
T_2r	99 3	4 4	5 1
T_2r^+	97 5	3 9	4 5
T r^+†	99 4	4 3	4 9
T ‡	99 9	5 5	6 3
T^+	97 8	3 2	3 7
T_7§	61 6	0 0	0 0

Th ty mi ute ubat t 37 C isot m b ff ed sal pt ted Mult p l c ty = 20

† 0 005 per c t DL t ypt phan dd d

‡ Inc l tion R ng s lut

§ I ub tio 0 002 M N Cl

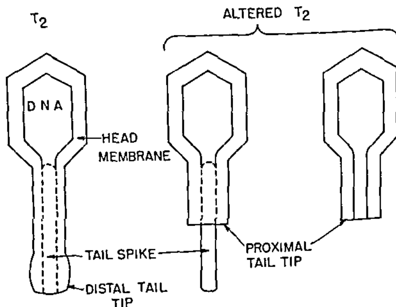


FIG 9 —Schematic representation of intact T_2 and altered T_2 phage with and without the tail spike

were prepared by treating phage with cadmium cyanide complexes and H O ethanol and by freezing and thawing These altered phage preparations digested cell walls under qualitatively different conditions (i e low ionic strength) than did intact phage particles (2) Furthermore T phages altered by freezing and thawing and by the action of cadmium cyanide complexes were able to digest cell walls of the mutant *E coli* B/2 which is completely resistant to the intact virus (Table 4) In addition frozen and thawed and $Cd(CN)_3^-$ treated T_4 preparations readily digested host cell walls in the absence of the co factor tryptophan which is needed for digestion by the whole T_4 phage (Table 5) It appears that the outside of the virus tail which contains a genetically controlled structure that adsorbs to the bacterial receptor not only is unnecessary for the action of the virus enzyme on the cell wall but must be removed before the enzyme activity can be demonstrated

TABLE 4

ACTION OF INTACT AND ALTERED T ON CELL WALLS OF *E c li* STRAINS B AND B/2

P A P ir	C W C R (P C)	
	E ol B (P p VII)	*E l* B 2 (P p 1)
Intact T r	4 3	0
$Cd(CN)_3$ treated T r+	8 9	6 7
Frozen thawed T r+	7 6	17 0

Several lines of evidence suggest that the active enzymatic site of the virus is the shortened proximal tail tip rather than the spike Most $Cd(CN)_3^-$ and frozen thawed T phage particles (2) have no spikes in the shortened tails After high speed centrifugation of these particles lytic activity is entirely in the pellet the supernatant fraction of frozen thawed T_2 virus containing the DNA and presumably some spikes is inactive Second T particles treated with H O ethanol under harsh conditions which leave relatively few spikes were found to be active while T_4 particles treated under mild conditions so that most of the spikes remained intact were enzymatically inactive (Table 5)

An important question which these experiments raised was the actual mechanism by which the phage tail was altered upon interaction with the cell wall The similarity between phage altered by zinc (or cadmium) cyanide complexes and phage altered by interacting with cell walls suggested that zinc in the cell wall was involved in this step of the infectious process It was possible to show

that the presence of zinc in the cell wall was necessary for the exposure of the viral enzyme and the digestion of the cell wall

It was found that each cell wall had about 3 100 atoms of zinc which were very tightly bound and could be removed only by extraction with 0 3 *M* trichloroacetic acid (TCA) The initial steps in phage cell wall interaction were found

TABLE 5

CO-FACTOR REQUIREMENT FOR CELL WALL DIGESTION BY INTACT AND ALTERED T_4 BACTERIOPHAGE

Preparation	Cell Wall Carb... Release (Per Cent)	
	Trypsin 0 5 Mg/Ml	No Trypsin
Intact T	3 9	0 6
Cd(CN)$_{3}^{-}$ treated T	3 7	3 5
Frozen thawed T	5 9	6 4
Mild H O EtOH treated T	0 0	0 0

TABLE 6

DIGESTION OF NORMAL AND ACID EXTRACTED [illegible] CELL WALLS OF *E coli* BY T AND T_4 BACTERIOPHAGE

Phage	[illegible] Cell Wall	Cell Wall [illegible] (Per Cent)
Intact T_2	Normal	1 8
Frozen thawed T	Normal	2 0
Intact T_2	Acid extracted	0 5
Frozen thawed T_2	Acid extracted	3 6
Intact T	Normal	4 3
Intact T_4	Acid extracted	0 2
Frozen thawed T_4	Acid-extracted	3 8

* [illegible] 0 05 mg/ml [illegible]

to be unaffected by the removal of the zinc from the cell wall Cell walls extracted with 0 3 *M* TCA when washed free of acid had lost none of their ability to adsorb bacteriophage even though they had lost most of their zinc

Since zinc was not involved in adsorption it was possible to test whether zinc was involved in the subsequent exposure of the viral enzyme The results of this

experiment are shown in Tables 6 and 7 Acid extracted cell walls were disrupted to only a slight extent by intact T_2 or T_4 phage The small amount of cell wall digestion caused by the intact phage can probably be attributed to small amounts of contaminant metal ions taken up by the cell walls when the acid was washed out On the other hand, T or T_4 phage which has been altered by freezing and thawing so that the normally concealed enzyme is exposed caused the release of C^{14} from both normal and acid extracted cell walls

Acid extraction of the cell walls removes not only the zinc but probably all metal ions from the cell walls Table 7 shows that only Zn^{++} restores the ability of intact T_2 phage to digest the cell walls Neither Cd^{++} nor Hg^{++} the other members of the zinc metal group (3) duplicate the action of zinc The amount

TABLE 7

EFFECT OF METAL IONS ON DIGESTION OF C CELL WALLS BY INTACT T BACTERIOPHAGE

C Cell Wall	Metal Added	Cell Wall C M d N ed m nt ble (Per C t)
Normal	None	[illegible] 4
Acid extracted	None	3 6
Acid extracted	$2\ ?\times10^{-7}\ M\ Zn^{++}$	3 7
Acid extracted	$4\ 5\times10^{-7}\ M\ Zn^{++}$	11 0
Acid extracted	$7\ 5\times10^{-7}\ M\ Zn^{++}$	14 4
Acid extracted	$4\ 5\times10^{-7}\ M\ Cd^{++}$	3 7
Acid extracted	$4\ 5\times10^{-7}\ M\ Hg^{++}$	4 1

Th trat f th m tal as th t prese t the fi l sol t Th m tal s n f t l salt) w e r n bated w th ll w ll t b ff f t h r t r m t mpe at e EDTA th dded t fi l con trat f 0 005 M a d th v us added I r

of C^{14} released in the presence of these metals is essentially the same as if no metal was added The addition of Ca^{++} Mg^{++} Co^{++} Mn^{++} or Fe^{++} to the cell walls did not allow their digestion by intact T_2 Further the small amount of Zn^{++} which restores the ability of intact T_2 to digest the zinc deficient cell walls is about what would be expected from the zinc content of cell walls The small amount of zinc necessary for the reaction also explains the relatively high blanks found with zinc deficient cell walls since some zinc is present in the solutions used to remove the acid after the extraction

The effect of zinc chelating agents on the disruption of cell walls by intact and altered T_2 phage was also measured (Table 8) The zinc chelating compounds markedly inhibited the digestion of cell walls by intact T but not by altered T_2 It can be concluded that the presence of a metal presumably zinc is necessary for digestion of cell walls by intact T but not by altered T_2

The effect of the same chelating agents (8 hydroxyquinoline and orthophenanthroline) on the infection of viable bacteria by T_2 phage is shown in Table 9. Bacteria pretreated with these two compounds were still viable, and the presence of these compounds did not interfere with the initial steps in viral cell interaction. The treated bacteria adsorbed T phage rapidly and were killed by

TABLE 8

EFFECT OF ZINC CHELATING AGENTS ON DIGESTION OF C CELL WALLS BY INTACT AND Cd(CN) TREATED T_2 PHAGE

T Ph g	C Mad N edimENT BLE (Per Cent)		
	None	8OHQ (0 008 M)	OP (0 00 M)
Intact	4 0	0 3	1 7
$Cd(CN)_2$ treated	4 0	4 9	5 7

8 OHQ = 8 OH qu l n OP = thophen throl Ch l t g t pr l ted th ll w l f t h t oom t mpe t f t rus pa t les dded

TABLE 9

EFFECT OF ZINC CHELATING AGENTS ON INTERACTION OF BACTERIA AND T_2 BACTERIOPHAGE

I et eatm nt f Host B t	T Ad bed† (Pe Ce t)	Host B te a K lled by Ph g (Pe Cent)	B ct I fected‡ (P C t)
None	98	99	60
0 008 M 8 OH quinoline	97	99	4
0 005 M orthophenanthroline	98	99	1

Host cell cul ted 20 m tes t room t mperat w th h lat g ge t N th h lat g mpo d affected host viab l t

† T d l l/host ll = 4 5

After 5 m t nc batio t is b ff

the adsorbed phage. However, practically none of the host cells was infected. These experiments clearly show that zinc is not functioning as a coenzyme for the viral tail enzyme. Phage altered in a variety of ways so that the enzyme is exposed readily digests zinc deficient cell walls in the absence of added metal ion.

Zinc has recently been shown to be a constituent of a large number of de

hydrogenases (21) and there is evidence that in bacteria the cytochrome system (22) and some dehydrogenases (23) are located in the cell membrane. It can be proposed that during bacteriophage invasion the bacteriophage tail comes in contact with a bacterial dehydrogenase in the cell wall which contains zinc and which has thiolesterase activity. This host cell enzyme ruptures thiolester bonds in the viral tail and exposes the viral tail enzyme. This is probably only the first host cell enzyme which the invading virus exploits for its own purposes during its reproductive cycle.

RELEASE OF VIRAL DNA FROM ITS PROTEIN COVERING

The forces involved in the release of viral DNA from within the viral head and its passage into the host cell are less well understood than the earlier phases of viral invasion.

T_2 altered by treatment with cadmium cyanide complexes has been used to study the release of viral DNA (5) on the assumption that these complexes essentially duplicate the action of the host cell wall. The intact T tail is 1 000 by 180 A, and in the center of the tail is a spikelike structure 100 A thick (10). After treatment with cadmium cyanide complexes the tail appears contracted and has dimensions of 500 by 260 A. Most of the particles have lost their center tail spike. It can be estimated that the passageway in the center of the tail of the altered phage particles which have lost their spikes is increased to about 140 A. This shortening and thickening of the tail to produce both a shorter and a wider passage would reduce the viscosity barrier and greatly facilitate the release of the threadlike DNA (diameter 22 A).

Special devices would seem necessary to overcome the problem of rapidly ejecting the DNA through this still rather narrow opening. From the results of these investigations in which one reaction controls or initiates a succeeding reaction (3 4), one would expect DNA release to be chemically controlled and that some compound(s) would initiate DNA release. It was found that various compounds could cause the rapid release of DNA from $Cd(CN)_3$ treated T (Table 10). The basic amino acids were the most effective in causing the release of viral DNA. Proline which does not have a primary amino group did not cause any release of viral DNA while glucosamine and tris hydroxymethyl aminomethane which do have primary amino groups were active. Even para amino benzoic acid had a slight activity. It can be concluded that a primary amino group is an essential feature of the structure of compounds which cause the release of the DNA.

It was also found that the release of DNA from altered T_2 phage was greatly affected by the pH at which the altered particles and test compounds were incubated. The release of viral DNA showed a maximum at pH 8 75–9 0 and at both lower and higher pH s less DNA was released. The release of viral DNA

was not greatly affected by lowering the concentration of the active compounds. For example, the amount of viral DNA released decreased only 50 per cent when the lysine concentration was lowered from 0.20 to 0.15 *M*. The configuration of the primary amino group did not appear to be important, since identical results were obtained with varying concentrations of L-lysine, as well as with DL-lysine.

The appearance of viral particles treated with $Cd(CN)_2$ and then with 0.2 and 0.02 *M* lysine at pH 9.0 is shown in Figures 10 and 11. When treated with

TABLE 10

EFFECT OF VARIOUS COMPOUNDS ON RELEASE OF DNA FROM T_2 TREATED WITH CADMIUM CYANIDE

Compound (0.2 M, pH 8.5)	T DNA Released† (Per Cent)
Arginine HCl	100
Lysine HCl	94
Ornithine HCl	57
Alanine	44
Threonine	32
Methionine	31
Isoleucine	30
Alanine	28
Aspartic acid	27
Cysteic acid	22
Histidine HCl	22
Glutamic acid	20
Glycine	17
Serine	8
Proline	0
Tris	97
Glucosamine	90
p-aminobenzoic acid	23
NH_4Cl	5
Borate	4

$T = 3$[illegible] [illegible] time = 30 minutes

† [illegible] per cent [illegible] the [illegible] buffer [illegible] 10 per cent [illegible]

0.2 *M* lysine, essentially all the particles have lost their DNA and the heads have a dimpled appearance. The electron micrograph field was chosen to include one intact T_2 particle for comparison. At a tenfold lower lysine concentration the particles can be divided into two groups: those which have apparently lost all their DNA and those which still retain their DNA within the head. The proportion of the two different particles agrees with the fraction of DNA released. It would appear that the release of the DNA from within the head and through the altered tail is an all or none phenomenon and that the partial release of viral DNA observed under various conditions is due to its release from a cor

responding fraction of the particles rather than to a partial release from all particles

All the compounds which cause DNA release (except tris) such as glucosamine and the amino acids, are normal constituents of the host cell wall It seems likely that the products of the action of the viral tail enzyme on the host

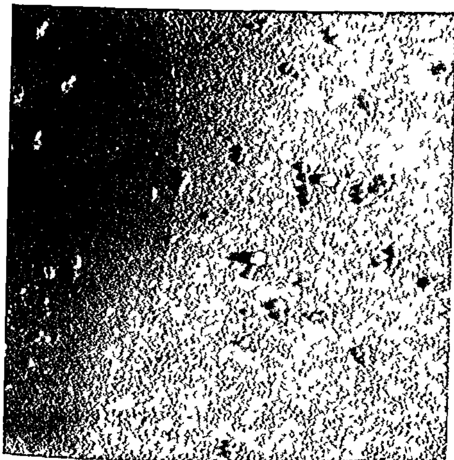


FIG 10—An electron micrograph of T phage treated with Cd(CN) and then with 0 2 M DL lysine buffer pH 9 0 One normal T_2 can be seen surrounded by short tailed empty headed particles 39 000×

cell wall which include peptides and other unidentified compounds (24) are the normal agents which overcome the barrier preventing the injection of viral DNA directly into the host cell

It might be proposed that the compounds which cause the release of the DNA from the opened tail of T_2 phage act by decreasing the attraction of the protein for the nucleic acid and increasing the repulsive forces between them At pH s

above 3, DNA will behave as a polyvalent anion Van Vunakis and Barlow (25) have reported that the only amino end group detectable in T protein (presumably head protein) is alanine The pKs for the amino group of alanine in simple peptides (26) range from 8 2 to 8 4 At neutral pH s it would be expected that some of the phosphates of the DNA would be neutralized by the positively charged amino groups of alanine but that as the pH is raised to 8 75 or above

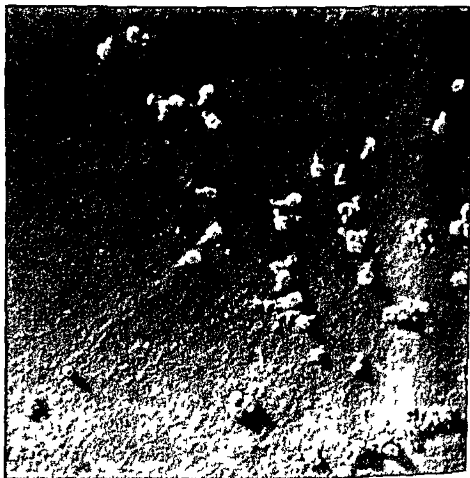


FIG 11 —An electron micrograph of T_2 phage treated with 0 02 M DL lysine pH 9 0 Approximately half the particles contain DNA and the rest have empty heads 8 000×

most of the alanine amino groups will be uncharged and the attraction between the protein and the DNA decreased The increased net negative charge on the protein (at pH 8 75 as compared to 7 0) would also act to repel the negatively charged DNA It is worth noting that one of the main products of cell wall breakdown by the viral tail enzyme is a small peptide containing diaminopimelic acid glutamic acid and a relatively larger amount of alanine (24)

CONCLUSION

The sequence of reactions which are thought to occur during bacteriophage invasion is illustrated in Figure 12 The virus particle adsorbs tail first to the host cell wall and the protein strands in the tip of the viral tail are partially unwrapped The host cell is killed at this stage of the interaction A bacterial zinc protein in the cell wall possibly a bacterial dehydrogenase hydrolyzes the exposed thiolester bonds in the virus tail which link the protein strands to the tail spike This causes a shortening of the tail and uncovers an enzyme which

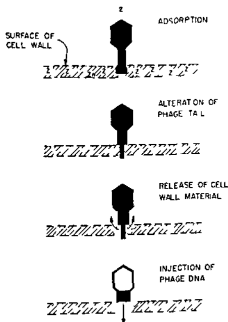


FIG 12 —Diagrammatic representation of the reactions occurring during bacteriophage invasion

digests a portion of the cell wall The products of this reaction are thought to trigger the injection of the viral DNA through the partially disrupted cell wall into the host cell

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CHAPTER 16

Functional Architecture of *Escherichia coli*

Richard B Roberts

FOR the past three years we have been attempting to uncover some of the processes used by micro organisms to assemble proteins and nucleic acids Here I shall report and interpret some of the experiments and then mention some of the speculations which provide fresh stimulus for further experiments I shall not limit myself to my own work rather I shall select various items from the work of our group [1]

S THE METABOLIC POOL

We started these studies with the knowledge that C^{14} labeled amino acids added to a growing culture of *Escherichia coli* appeared shortly thereafter in the proteins The time required for incorporation was so short that it was necessary to devise a new technique for taking samples (Fig 1) With the arrangement shown it is possible to take samples rapidly enough to follow the kinetics of the process

Figure 2 shows the typical time course of incorporation of an amino acid The radioactivity builds up first in the trichloracetic acid (TCA)-soluble fraction and subsequently appears in the insoluble fraction The kinetic relationships which are revealed indicate that there is a transient metabolic pool and a kinetically stable end product Similar experiments have demonstrated the following properties of the pool

1 Pool formation is a process distinct from protein synthesis pools are formed even though protein synthesis is blocked

2 Pool formation is strongly inhibited by lack of an energy supply however the exchange of external amino acids with an existing pool is much less depend

1 The Biophysics Section Ellis T Bolton Roy J Britten Dean B Cowie and Richard B Roberts staff members John J Leahy Carnegie Fellow and Frank T McClure special Carnegie Fellow on leave from Applied Physics Laboratory Johns Hopkins University

POROUS COLLODION FILTER

EVACUATED

CONSTANT TEMP BATH

TCA SUSPENSION ALSO FILTERED

10% TCA

EXPERIMENT INITIATED BY VIGOROUS INJECTION OF LABELED AMINO ACID

SAMPLES WITHDRAWN ALTERNATELY TO MEASURE TOTAL UPTAKE OR INCORPORATION INTO PROTEIN

FIG. 1 — Apparatus used for rapid sampling to measure kinetics of pool formation. TCA = trichloracetic acid.

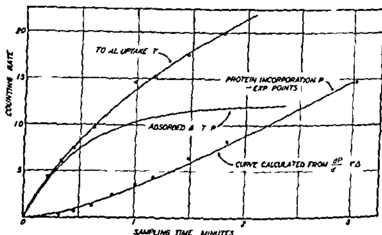


FIG. 2 — Adsorption and protein incorporation of C^{14} proline by *E. coli* cells pretreated with C^{12} proline. C^{12} proline (0.8×10^{-4} molar) added 1 minute before the carrier free C^{14} proline. An amount of medium was added with C^{14} proline such that there was no change in proline concentration. Concentration of cells 0.2 mg(dry)/ml

ent on the energy supply (cf Fig 3) The rate of loss from an *existing* pool is very low, even in the absence of an energy supply

3 The pool size is proportional to the external concentration of the amino acid over a wide range of concentration but finally reaches a saturation value (Fig 4) The concentration ratio (internal/external) for proline is more than

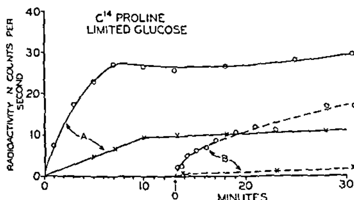


Fig 3 —Maintenance and exchange of pool proline in the absence of glucose In both experiments growing cells were suspended at time zero in medium containing 10 γ/ml glucose and 0 87 γ/ml C proline In experiment *A* a small quantity of C14 proline was added at time zero in *B* an equal quantity of C14 proline was added at 13 minutes In each case the upper curve (*circles*) represents the total C14 proline taken up and the lower curve (*crosses*) the C14 incorporated into protein The difference is the C14 proline in the pool

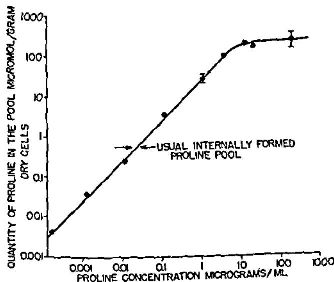


Fig 4 —Quantity of C14 proline taken up into the pool as a function of the concentration of C14 proline added to a growing culture of *E coli*

500 to 1, and the saturation pool size is 240 μmoles/gm dry cells Other amino acids give different concentration ratios and different saturation pool sizes

4 Pool formation is specific to a limited degree the formation of small pools of proline and methionine is not affected by the presence of other amino acids however large pools of proline cannot be formed when other amino acids are present In contrast some groups of amino acids show competition during pool formation—leucine isoleucine and valine for example mutually interfere at all levels In this case it can be seen that the specificity of the protein forming mechanism is greater than the specificity governing pool formation (Fig 5)

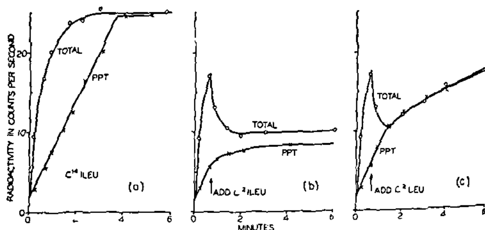


FIG 5—Leucine isoleucine interaction *a* Control 0 3 γ/ml C^{14} isoleucine added at time zero to 0 25 mg/ml of growing cells (dry weight) *b* Effect of carrier isoleucine Same as *a* with 10 γ/ml C^{12} isoleucine added at 40 seconds *c* Effect of leucine competitor Same as *a* with 10 γ/ml C^{12} leucine added at 40 seconds Note that rate of incorporation into protein is not affected until the pool is completely depleted

5 The pool material is in the direct line of protein synthesis since the rate of incorporation of radioactivity into protein is proportional to the specific radioactivity of the pool amino acid even when the specific radioactivity of the pool is quite different from that of the medium

6 Paper chromatography of the material extracted by TCA or 80 per cent ethanol shows purine and pyrimidine nucleotides glutathione and free amino acids as the principal compounds When a radioactive amino acid is added to the culture medium, the radioactivity of the soluble fraction is found in the amino acid supplied and in closely related amino acids for example valine is partly converted to leucine, but proline and methionine show little conversion No small peptides are observed even when protein synthesis is blocked and peptides might be expected to accumulate

7 The pool is removed by osmotic shock (Fig 6), but no permanent damage

is done to the cell, and the pool is rapidly re formed (Fig 7) The pool can also be removed by hydrostatic pressure

8 The saturation level of the pool however depends on the osmotic strength of the growth medium

9 The kinetics of the incorporation of pool material into protein demonstrate that the formation of a peptide chain is a very rapid process Figure 8 shows that there is no kinetic delay in excess of that required to build up the specific radioactivity of the pool Chemical tests indicate that the radioactive amino

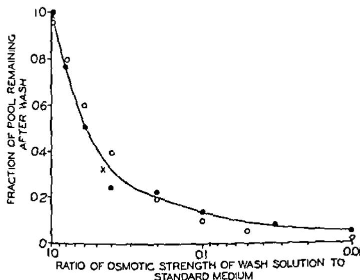


Fig 6 —Removal of pool by washing cells with solutions of low osmotic strength Growing cells were supplied C^{14} proline and allowed to form a pool then samples were collected on a membrane filter Various wash solutions were passed through the cell pad on the filter *Solid circles* cells washed with various dilutions of the standard growth medium *open circles* with NaCl solutions *crosses* with glucose solutions

acids incorporated into the TCA insoluble fraction during 15 seconds are truly linked into long peptide chains and not merely concentrated in the end groups It is estimated that a 3 second kinetic delay would have been observable accordingly we are led to believe that only a few seconds at most are required to complete the formation of a peptide chain

Amino acids used in these experiments include proline methionine valine tyrosine and alanine Some experiments were done with a mixture of amino acids from hydrolyzed *Chlorella* protein The amino acids were not all tested individually (Further details of the experiments can be found in references 1 2 and 3 general methods are described in reference 4)

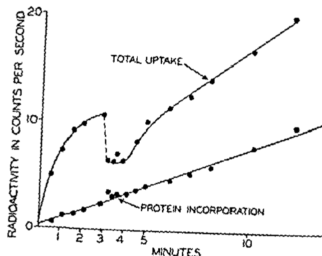


FIG 7 —Recovery of pool after removal due to reduction in osmotic strength At time zero 0 3 γ ml C^{14} proline was added to 0 08 mg ml of growing cells (dry weight) At 3 minutes 2 volumes of water containing 0 3 γ ml C^{14} proline were added

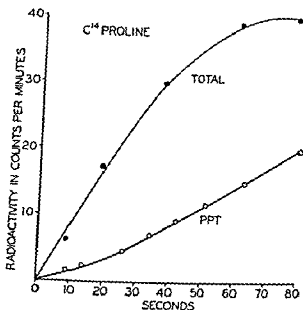
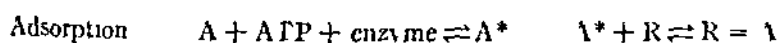


FIG 8 —Carrier free C^{14} proline was added to a culture of *E coli* growing at 18 The incorporation into the TCA precipitate reaches its full rate in 25 seconds The observed kinetic delay is due to the time required for pool formation

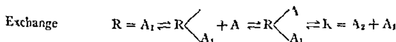
I. ADSORPTION SITES

These properties of the pool can be interpreted in terms of an adsorption process. According to this model a concentration gradient between the cell and the medium is maintained because the amino acids of the pool are not free within the cell but are adsorbed to larger molecules. The requirement for energy to enlarge the pool indicates that an activating mechanism such as the amino acid-activating enzymes (5, 6) operates to supply energy needed for adsorption. The rapid exchange and slow loss are characteristics which might be expected in a multipoint attachment of amino acid to macromolecule. Such reactions are indicated below.



Adsorption $A + ATP + \text{enzyme} \rightleftharpoons A^*$ $A^* + R \rightleftharpoons R = A$

(A = amino acid, A^* = amino acid in activated state, R = adsorption sites on macromolecules),



Exchange $R = A_1 \rightleftharpoons R\langle_{A_1} + A \rightleftharpoons R\langle^{A}_{A_1} \rightleftharpoons R = A_2 + A_1$

(A_1 and A_2 represent different molecules of the same species; exchange may also proceed by reversal of the adsorption reactions).

The activating enzymes undoubtedly contribute to the specificity which is experimentally observed during pool formation. The interference among members of the leucine group at concentrations far below those required to approach saturation is a strong indication of competition for the activating enzyme. However, there is probably some additional specificity in the sites themselves.

The nature of the sites is not yet well defined. The cells contain roughly 600 µmoles of ribose nucleotides per gram dry weight. If all this material were to act as templates for protein synthesis and if 3 nucleotides were required to specify a site for one amino acid, then there might be specific sites available for 200 µmoles of amino acids. Of these, 5 per cent might be expected to be proline sites since proline makes up 5 per cent of the protein. Accordingly, there might be 10 µmoles of proline sites, enough to accommodate a small proline pool but far too few to hold the large pools observed when proline is supplied at higher concentration. With media of high osmotic strengths the proline pools may reach 1,000 µmoles/gm dry cells and may exceed even the total quantity of nucleotides. In this case it is evident that other molecules (perhaps proteins) must also provide sites. The specificity of the latter sites is evidently low, as the large proline pools do not accumulate when other amino acids are present. Accordingly, it is necessary to consider the sites not as a homogeneous array but as heterogeneous sets of sites ranging perhaps from template sites of high specificity and high affinity to much less specific sites of lower affinity.

In the yeast *Candida utilis*, it is possible to demonstrate two types of pools (7, 8) When nucleic acid bases are supplied in small quantities, they are phosphorylated and held in a nucleotide pool When supplied in higher concentrations they are accumulated also as free bases These chemically distinct pools can also be distinguished by kinetic measurements the small pool consisting of several nucleotides, is used for nucleic acid in preference to the large pool of free bases

The same kinetic distinctions can be made when the yeast accumulates amino acid pools In this case however, no chemical differences have been found and the kinetic difference may be taken as an indication that a variety of different sites is present In *E coli* there are some indications that the pool is not homogeneous but exchange is much more rapid in the bacterium than in the yeast and differences among amino acid-binding sites cannot be clearly demonstrated

The osmotic sensitivity of the pool gives another hint as to the nature of the sites It is evident that osmotic shock produces a profound but temporary effect on the binding Such a result might be expected if the binding sites were associated with osmotically sensitive structures and if the different points of attachment moved during the disturbance caused by osmotic shock Possibly the amino acids form bridges between different strands of protein or nucleic acid or between adjacent coils of a folded strand

The site model seems adequate to give a qualitative interpretation of the properties of the pool In reaching this interpretation, the following features were incorporated into the model an activating mechanism a multiplicity of different sites a multipoint attachment of the amino acids and an osmotic sensitivity of the sites Further work determining temperature coefficients of adsorption desorption and exchange shows promise of yielding a reasonably complete quantitative interpretation of the observed facts

Experiments similar to some of those described above have been carried out at the Pasteur Institute (9 10) While there is no conflict in the experimental results the Pasteur group has given a quite different interpretation They attribute the maintenance of concentration gradients to permeability barriers which the amino acids penetrate with the help of permease enzymes This model may also be capable of yielding a qualitative interpretation of the facts A final choice between the two will probably depend on the success of one model or the other in yielding a quantitative interpretation of the data

V LOCATION OF THE POOL

These considerations of the observed properties of the pool and the different interpretations which can be placed upon them lead to speculation as to how the structure of the cell might be related to its function What structures of the

cells might provide osmotically sensitive sites? Or what structures of the cell provide the impermeable regions needed in the permease model

The most obvious structure of *E coli* is its cell wall There are a number of experiments however, which indicate that the cell wall does not offer much resistance to diffusion When large pellets of cells are mixed with solutions of radioactive amino acids (under conditions designed to minimize metabolic effects or pool formation) the pellet is roughly 50 per cent as effective as an equal volume of water in diluting the solution As the intercellular water of the pellet accounts for only 20 per cent of the pellet volume it appears that the amino acids must penetrate through the cell wall The amino acids which enter the cells under these conditions are also free to diffuse out these thick suspensions when diluted and filtered lose their amino acid content in a time less than the few seconds required for filtering (2, pp 118–20)

Moreover the cell wall can be weakened by treatment with lysozyme without affecting the pool size If lysozyme treated cells are given a severe osmotic shock, they lyse when the shock is somewhat less severe they swell up into round forms but can still accumulate a small pool Quite large molecules (sucrose) must be used as solutes to produce osmotic shocks effective in rupturing the cell walls, smaller molecules seem to penetrate too freely

Nucleoprotein particles of the cell provide another structure which might be impermeable to amino acids or osmotically sensitive There is considerable evidence to show that these particles play an important role in protein synthesis (11) consequently it would be a very fortunate arrangement if the particles could also hold the amino acid pools Sedimentation analysis shows that there are three types of particles in *E coli* roughly 20s 26s and 40s The 40s particles have a molecular weight of roughly 10^6 and are 50 per cent protein and 50 per cent RNA The diameter of the hydrated particle is approximately 200 A (12 13 14) The average molecular weight of the nucleotides of RNA is 325 accordingly there are 1,540 nucleotides per particle giving a total chain length of 2 600 A if the RNA is considered as a double strand Correspondingly there are 4 530 amino acids per particle giving a total peptide chain length of 16 700 A

If the protein were localized on the surface of the particle the spacing between peptide chains would be 7 A Alternatively the protein could be arranged in coils with the axes parallel to the surface In either case the spacing is close enough to provide an osmotic barrier and the possibility of sites which would let the amino acids bridge between two strands The concept of an osmotically sensitive structure of the microsomal particles would also be helpful in understanding the temporary cessation of protein and nucleic acid synthesis which occurs when the cells are suddenly placed in a medium of increased osmotic strength

The total volume of the particles can be estimated Centrifuge runs show that most of the cell s RNA and 30–40 per cent of the protein can be spun down in the particle fraction The total material is thus roughly 40 per cent of the dry weight and might be expected to comprise approximately 40 per cent of the wet cell volume This estimate of the volume of the particles corresponds roughly to the 50 per cent of the cell volume into which amino acids do not diffuse

This view of the cell particles as likely sites for amino acid adsorption has led us to a number of attempts to isolate the particles with the amino acid pools still intact Various methods which have been tried include differential cen trifugation chromatography, and electrophoresis To date we have succeeded in keeping only a very small fraction of the amino acid pool associated with the particles Perhaps further studies will indicate what conditions are required to maintain the stability of the particles after they are removed from the cells

The number of particles can also be calculated Assuming that all the RNA of the cell is in the 40s particles and taking 1 5 $\times 10^{10}$ as the total molecular weight of RNA in a small cell there would be thirty thousand 40s particles per cell However this calculation neglects the 20s and 26s particles which appear in the sedimentation diagram These smaller particles seem to contain less RNA than the 40s particles as short preparative centrifuge runs give pellets which are richer in RNA also the total protein of the pellets is higher than would be expected from 40s particles alone Assuming that the 20s and 26s particles have one fourth and one half the RNA content of the 40s particles and that there are equal numbers of each class the total number of particles is about fifty thousand per cell

The total molecular weight of protein in such a cell is 6 $\times 10^{10}$ the rate of protein synthesis is 2 per cent per 100 seconds or 10^3 peptide chains per second (MW 13 000 125 amino acids of average MW 122) If we assume that the 40s particles are responsible for protein synthesis, then each particle mu t synthe size one peptide chain per 17 seconds Since kinetic measurements show that the time required for the completion of a peptide chain is less than 3 seconds it seems possible that each 40s particle accumulates amino acids until it has the entire supply required for a complete peptide chain and then the peptide bonds are formed

According to this picture the 40s particles would contain on the average half the amino acids required to complete a peptide chain (as a minimum for amino acids in short supply) Six molecules of proline are required to form the average peptide of 125 amino acids accordingly, the minimum content of pool proline should be 50 000 per cell or 0 5 μmole/gm dry cells This is the value observed when the cells grow with glucose as the sole carbon source

While these speculations on the possible role of particles holding pools are largely unsupported by facts, they do provide a picture which relates the struc

ture of the cell to its functions, and, more importantly they suggest new experiments

G THE GROWTH OF PARTICLES

The particles make up such a large fraction of the cell that to a first approximation the problem of duplicating a cell is the problem of synthesizing enough particles for a new cell Let us then guess that the three types of particles observed in the analytical centrifuge represent three phases in the growth of a particle Since the smaller particles seem to have a lower RNA content we assume that they grow by RNA synthesis and that the RNA content of the three classes is respectively, 1 2 and 4 molecules The 1 540 nucleotides of the 40s particle are visualized as 4 molecules of 385 nucleotides each We then assume that the 40s particle has accumulated enough RNA that protein synthesis can begin Taking three bases to specify one amino acid there is sufficient information in the 4 RNA molecules to determine the order of a peptide chain of 128 links As protein synthesis proceeds, we assume that the 40s particle accumulates protein, growing larger and less stable until it falls apart into four 20s particles and non particulate protein

This model provides an organization of known facts and it has already stimulated experiments to look for kinetic relationships among the particles To date we can report only that after a short exposure to radiosulfur the protein fractions obtained by differential centrifugation do indeed show different specific radioactivities Only minor improvements in the techniques of separating particles are needed to test this hypothesis and to provide the data to indicate how it should be revised

G ORGANIZATION OF THE PARTICLES

In this model each particle contains and synthesizes only one variety of protein It is known however, that many of the enzymes occur in the particulate fraction and that the enzymes are organized into enzyme systems It then becomes necessary to provide a spatial organization of the particles As the cell wall does not have sufficient area to hold the particles we have chosen the other large component of cells DNA to provide an organizing framework Taking a molecular weight of $6\,5 \times 10^6$ for DNA the length of a Watson Crick spiral is $3\,5\,\mu$ To fit into half of a small bacterium this molecule must be coiled to reduce its length by a factor of 5 Choosing 200 A for the pitch of the spiral the diameter must be 300 A Such a spiral will accommodate 35 large particles (1 per turn) on the inside with the associated small particles held on the outside Substrate molecules could proceed from one particle to the next by surface diffusion

The 580 DNA molecules per cell then provide 20 000 sites for each class of particle A complete structure might consist of an array of 290 DNA spirals

with their axes parallel Its cross sectional area is two thirds the cross section of the cell One such array would fit into each end of a small cell giving a cell which is essentially two nuclei surrounded by a cell wall

This model is suggestive in a number of ways For example, it provides a reason for the correspondence between the order of the genes and the sequence of biochemical events which is sometimes observed It suggests that exogenous DNA might be pulled into the cell by the attachment of particles which might have a ratchet like action Once inside the cell foreign DNA such as virus might affect the metabolism by rearranging existing enzymes It suggests a large scale order within the cell which might be observable by X ray diffraction

It is possible to imagine a way in which such a structure could duplicate If the 40s particles accumulate protein and disintegrate they could at that time lose the attractive forces holding them to the DNA spiral The DNA would then be free to unravel and duplicate In the meantime the two smaller particles have grown into two 40s particles which are ready to attract the two newly formed DNA strands In this way the original structure would be duplicated in detail However it would then have twice its original cross section making it too large to fit comfortably within the cross section of the cell This situation would bring into play compressional forces which might cause one half of the structure to extrude from the other half by a longitudinal motion along the axes of the spirals

In conclusion I should like to emphasize that I have presented some experimental facts some interpretations of these facts and some pure speculation To avoid any possible confusion between fact and fancy the various sections are labeled S L V and G standing for solid liquid vapor and gas These different categories must be judged by quite different criteria In particular the speculative models need only to avoid contradiction of known facts to organize known facts in a form that is easy to remember and to suggest new experiments

Note added in proof—Knowledge of the particles has advanced so rapidly that reading this paper is like visiting a museum Many of the details of the speculation regarding the particles have already been shown to be wrong *It is* of interest however that the hypothesis that the smaller particles are the precursors of larger particles has been very useful and is now supported by a number of experiments A more recent and more accurate summary of information on particles is given in *Microsomal Particles and Protein Synthesis* (New York Pergamon Press 1958)

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CHAPTER 17

Biochemical Aspects of Natural Resistance toward Infection

Herbert Fischer

ALMOST every kind of micro organism will grow in a medium composed of amino acids, proteins glucose, electrolytes vitamins and some trace elements This is the composition of our extracellular and intracellular body fluids, and yet it is only relatively rarely that while we are alive our body fluids become the culture medium for the growth and multiplication of bacteria and viruses It is obvious that there must be mechanisms to prevent bacterial invasion and to destroy micro organisms within our blood stream and tissues Moreover these mechanisms must differ from the *specific* immunity provided by specific antibodies Antibodies are formed in response to specific antigens and do not appear in the blood stream until the sixth or seventh day following the first injection of antigen This time lag in the absence of other defense mechanisms, would allow an enormous increase in the number of micro organisms The forces or mechanisms which comprise the phenomenon of *natural resistance* or *natural immunity* are *non specific* these forces are always present, since they are able to start their action at once

In 1880 Metchnikoff described the phenomenon of *phagocytosis* by the microphages, identical with the polymorphonuclear white blood cells, and the macrophages as present in lymphatic and connective tissue as part of the system later called 'RES' (reticuloendothelial system) In 1889 Buchner found that blood fluid even in the absence of cells capable of engulfing and digesting bacteria exerts a remarkable power to destroy bacteria, he termed the hypothetical and unknown bactericidal forces in serum alexins' Interestingly enough although these two main principles of non specific resistance—phagocytosis and alexins—have been known for almost eighty years only recently have biochemists become seriously engaged in the elucidation of the underlying molecular problems One of the reasons that this interest has arisen so suddenly is the observation that exposure to an overdose of radiation is followed by a

marked decrease in natural resistance On the other hand the main reason for the long lasting neglect of this field is the fact that to many biologists and chemists the development of powerful chemotherapeutic agents and antibiotic seemed to be much more promising

In this chapter I should like to discuss, first the bactericidal principles present in our blood fluid and then go on with a discussion of some aspects of phagocytosis As a third point, the possible mode of action of antibiotics of our own body will be outlined

THE PROPERDIN SYSTEM

A recent, and perhaps the most fruitful line of investigation in non specific defense mechanisms of the body is the properdin system It was known that Buchner s alexins are inactivated by heating serum to 56° C for 20 30 minutes Many workers suspected that a close relationship existed between alexin and serum complement which has a thermolabile component and can be inactivated at the same temperature However, it remained for Louis Pillemer (1) to demonstrate clearly that, in addition to the four known components of complement designated as C 1 C 2, C 3 and C 4 there exists a fifth factor which is essential for the greater part of the bactericidal properties of serum Pillemer named this factor properdin (from Latin *perdere* to destroy) Since properdin acts only in the presence of complement and Mg^{++} ions all these components are now included in the properdin system

Properdin is a euglobulin and contains lipid carbohydrate and phosphorus It is not an antibody and its action is non specific it requires complement C and Mg^{++} ions for activation Properdin can be removed from serum at 17° C by adsorption on zymosan an insoluble polysaccharide complex in yeast cell walls On removal of the properdin zymosan (PZ) complex by high speed centrifugation the remaining serum (RP serum) possesses all four components of complement but is practically devoid of bactericidal activity When properdin or the PZ complex is added activity can be completely restored

The PZ complex can be dissociated by solutions of neutral salts of rather low ionic strength (0 6 M NaCl) which suggests a relatively loose combination After dissociation properdin can be purified by dialysis and ultracentrifugation and then dried from the frozen state It is a rather stable protein with a molecular weight of about 2 200 000 On paper electrophoresis it migrates with the gamma globulin fraction In human serum it is present normally in a concentration of about 30 mg/100 ml

An interesting feature of the properdin system which gives some clue to its mode of action is that treatment of serum with zymosan below 20° C will remove properdin and leave complement intact At higher temperatures the PZ complex combines with complement, thereby inactivating component C 3

This process which tentatively leads to the activation of an enzyme requires Mg^{++} ions

In order to give a rough idea of the role of properdin, we might compare it to a primordial antibody which is able to form a complex with membrane substances of bacteria and viruses and under certain conditions even with the walls of our own body cells The complex thus formed is available for the lytic action of complement However there are distinct differences in lytic actions as brought forth by the properdin system on the one hand and the antigen antibody reaction on the other Specific antibodies do not require Mg^{++} ions to form a complex with the corresponding antigen Once formed antigen antibody

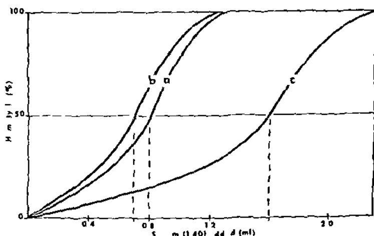


FIG 1 —Principle of a properdin determination Each curve represents a complement titration of serum by means of sheep erythrocytes and amboceptor the degree of hemolysis is read photometrically (546 mμ) and the 50 per cent value is selected for use in quantitative comparisons *a* Test system without properdin and with C 3 as limiting component *b* same system plus serum to be tested for properdin *c* same system as *b* after treatment with zymosan at 37 C for 1 hour The decrease in C 3 titer brought about by passing from *b* to *c* is a function of the properdin content of the serum tested

aggregates will inactivate components C'2 C'1 and C 4 but will not remove C 3 In contrast to this properdin complexes require the presence of all four components but will inactivate only C'3 The decrease of C 3 activity is proportional to the amount of properdin present According to Pillemer (2) 1 unit of properdin corresponds to the inactivation of 120 units of C'3 in 1 ml of serum

The determination of properdin based on these principles is simple (Fig 1) However the difficulties lie in the preparation of test sera with high complement titer having C 3 as the limiting component We have been fortunate in finding that guinea pig serum as a test system has definite advantages over human serum which is used by other workers

PROPERDIN LEVELS IN EXPERIMENTAL AND PATHOLOGIC CONDITIONS

Although many questions concerning the synthesis kinetics and mode of action of properdin have as yet been unanswered its biologic importance has been demonstrated in various fields Guinea pigs are very susceptible to infections and have a low properdin titer In contrast rats which are well known for their resistance show the highest titer Man and the rabbit have an intermediate position One may speculate whether these species differences reflect variations in natural resistance Total body X irradiation as has been found by Ross (3), results both in a profound drop in the properdin level and in fatal bacteremia in mice Brues and Stroud (4) showed that at least part of the radiation damage can be prevented by the injection of Cohn s serum fraction III which contains properdin

We have studied the properdin levels in patients in our clinic in Frankfort (5) We were able to show that immediately following various stress situations there is a marked and sharp drop in properdin activity This is accompanied by a decrease in leukocyte count an increase in the fibrinolytic activity of plasmin and a decrease in complement titer to mention a few manifestations of the *vegetative Gesamtumschaltung* (6) We were able to demonstrate that this drop in properdin activity occurs not only in typical shock but also after periods of maximal muscular activity (early postpartum) Most significant was the drop which occurred after diagnostic encephalography (Fig 2) a procedure in which part of the cerebrospinal fluid is replaced by air (5) I should like to point out that this initial drop of properdin with concomitant decrease in the bactericidal activity of the serum may well account for the fact that many types of stress situations enhance the start of latent infections

We feel that this drop in properdin is very probably caused by the release of heparin from mast cells in blood and connective tissues Since heparin is a polysaccharide, it can act like zymosan Small amounts of heparin in vitro can inactivate properdin and the second component of the complement With human patients the amounts of heparin released in stress situations are too small to be detected by our present techniques However in experimental peptone shock in dogs Dr Fritzsche in our laboratory could clearly demonstrate that with heparin release there was a decrease in properdin (Fig 3) in this instance the in vivo and in vitro studies correspond

Heparin is not the only substance capable of reacting with the properdin system Lipopolysaccharides as present in cell membranes of various viruses bacteria and cells can be regarded as zymosan like substances Normal erythrocytes or stromata do not react with the properdin system but after treatment with tannic acid or digitonin they do This indicates that injured or abnormal cells under certain conditions become available to the lytic action of properdin and complement This has been definitely established for the abnormal erythro

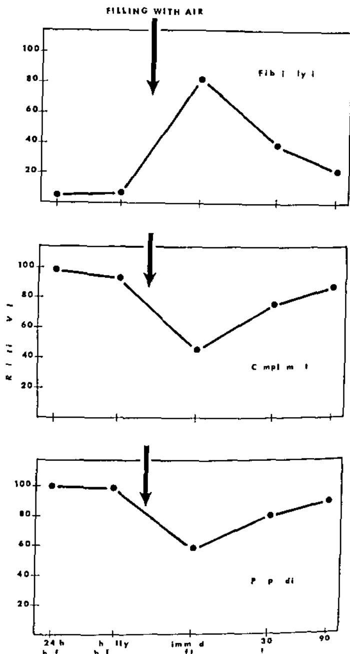


FIG 2.—Behavior of fibrinolysis, complement and properdin in relation to the introduction of air in encephalography. Plotted values for fibrinolysis and complement are averages of 12 patients; those for properdin, 6 patients.

cytes present in Marcia Fava anemia a rare disease which leads to nocturnal hemoglobinuria

Thus properdin, in addition to its virus neutralizing and bactericidal properties appears to have another important function in our organism It may participate in the clearing of cellular debris (worn out cells) to speak in current 'Chicago terms, it has some slum clearing function For example in wide spread necrosis in postoperative complications, or in certain patients with carcinoma this may lead to the release of toxic proteolytic breakdown products

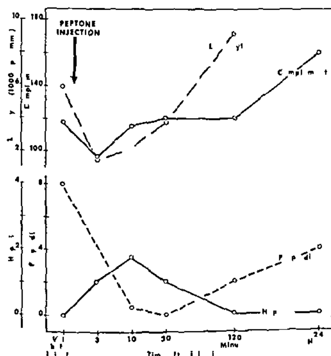


Fig 3 —Relations among properdin heparin complement and leukocyte count in a 29 kg dog injected with 21 ml of 10 per cent peptone solution

from the clearing area and might conceivably cause shock and kidney damage One must be cautious about predicting that properdin is an ideal substance for the treatment of all types of infectious diseases

Thus far, all attempts to make properdin available for substitution therapy have failed mainly because most preparations from animal sources have been antigenic At present the most promising way to increase the properdin titer seems to be the intravenous injection of purified endotoxins of bacterial origin These well characterized lipopolysaccharides are most interesting in that relatively few molecules will initiate the entire series of systemic body responses

starting with fever increased fibrinolytic activity decreases in leukocytes complement and properdin etc Some hours later, the complement and properdin titers rise above the initial level and considerably increase resistance toward infection over a period of several days Animals after such a treatment survive an otherwise lethal infection (2)

Working with patients and using Otto Westphal s ' Lipopolysaccharid from bac ab equi we consistently found that 24 hours after the injection of 0 5 γ Lipopolysaccharid the properdin titer in the serum was increased by a factor of 2 or 3 (Fig 4)

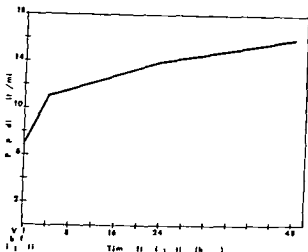


FIG 4 —Average course of changes in properdin content after injection of bacterial lipopolysaccharide (Pyrexal 0 5γ) into patients

PHAGOCYTOSIS

Phagocytosis can be dealt with here only from a very narrow angle Most of the innumerable papers published since Metchnikoff s discovery have treated the phenomenon from a physicochemical standpoint explaining the engulfment of particles or bacteria as a *passive* phenomenon, which depended upon the ratio of cell/particle surface tension On the other hand Hamburger as early as 1912 had found that sodium fluoride is a potent inhibitor of phagocytosis which indicates that *active metabolic forces* are involved The effects of some metabolic inhibitors on phagocytosis are shown in Table 1 As is evident inhibitors of glycolysis (fluoride iodoacetate) inhibit the uptake of bacteria whereas cyanide and dinitrophenol have no effect These findings may be readily explained by the fact that mature polymorphonuclear leukocytes have a metabolism similar to embryonic and tumor cells i e, characterized by a high rate of aerobic glycolysis

The next point of biological importance is the metabolism of a phagocyte after it has engulfed a bacterium or an inert particle. We have done some experiments along these lines (7). Sedimented hog leukocytes were washed in silicone treated vessels and were then introduced into Warburg flasks. After a period of time heat killed bacteria were added from the side arms. After the

TABLE 1

PHAGOCYTOSIS BY LEUKOCYTES AFTER INCUBATION WITH METABOLIC POISONS

Metabolic Poison	Per Cent Phagocytes	Inhibition (Per Cent)
None (Ringer's solution)	85	
Sodium fluoride 0.02 M	12	86
Iodoacetic acid 0.005 M	24	72
Sodium cyanide 0.01 M	90	0
2,4-Dinitrophenol 0.0005 M	79	7

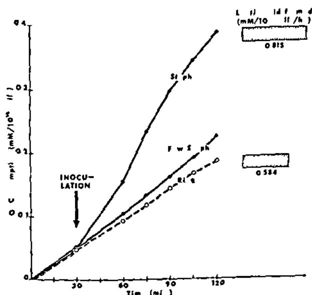


FIG. 5.—Metabolism of leukocytes as influenced by addition of staphylococci. After Ic. glucose 100 mg per cent.

addition of bacteria the oxygen consumption (here expressed in millimoles per 10^{10} cells) increased immediately (Fig. 5). This increase was about 10-20 per cent when only 2-3 bacteria per single leukocyte were added but was 100-200 per cent when larger quantities (10-20 bacteria per single leukocyte) were added. The concomitant increase in lactic acid production (Fig. 5) is not so

impressive but one must consider that the cell suspensions had to contain about the same number of erythrocytes in order to prevent sticking and clumping of the white cells These red blood cells produce a considerable amount of lactic acid With this in mind one can see that the increase in lactic acid production is significant A pure suspension of erythrocytes or of Ehrlich mouse ascites tumor cells which cannot phagocytose is not affected, either in respiration or in glycolytic rate by the addition of bacteria

When India ink is phagocytosed there is a large increase in lactic acid formation but practically no increase in oxygen consumption (Fig 6) It was assumed that this difference in metabolic response was the result of the bacterial sub

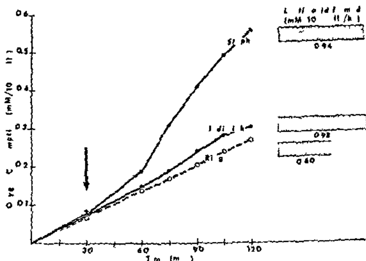


Fig 6 —Comparative influence of India ink and staphylococci on leukocyte metabolism Aerobic glucose present

strate since India ink is metabolically inert We treated the bacteria with formaldehyde and then boiled them in alcohol for several hours Despite this denaturation, there was a marked increase in glycolytic rate and respiration The difference in the response evoked by bacteria and India ink is still unexplained

An increase in oxygen consumption takes place even in cyanide treated leukocytes when bacteria are added (Fig 7) This excludes the possibility that electron transfer is conducted through the cytochrome system as present in the digitonin fragments of mitochondria Dr Lehninger, who was kind enough to discuss the phagocytosis problem with me, suggested that an increased transfer from cytochrome b_5 directly to oxygen might take place this is comparable to other stimulated or induced cells Cytochrome b is located in the cytoplasm and cannot be blocked by cyanide

Stahelin Suter and Karnovsky (8) from the Rockefeller Institute have recently published some observations on the interaction between phagocytes and tubercle bacilli Using exudate leukocytes from the peritoneal cavity of guinea pigs they found in accordance with our findings and some earlier ones an increase in oxygen consumption but were not able to detect a corresponding increase in glycolytic rate This discrepancy deserves further study but will probably be explained by differences in cellular material and in technique

Thus far all we have learned from our studies with leukocytes is that these cells are especially endowed with the potentiality for sudden changes in their enzymatic activities However this does not explain either how living bacteria

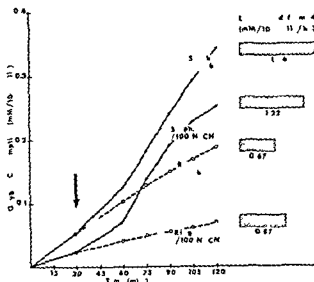


FIG 7 —Influence of staphylococci on metabolism of cyanide treated leukocytes

are actually killed inside the cell or how they are made available for enzymatic attack in an area of inflammation

BACTERICIDAL SUBSTANCES FROM LEUKOCYTES

It has long been suspected that leukocytes contain bactericidal substances which are eventually released at the site of inflammation The first such substance a lysozyme like mucolytic enzyme was discovered by Fleming (9) Its bactericidal activity is restricted to some rather harmless Gram positive bacteria it plays no significant role in natural defense Another bactericidal protein phagocytin has recently been described by Hirsch (10) It has been extracted from the cytoplasmic fraction of polymorphonuclear cells and is active against many Gram negative and Gram positive bacilli Its mode of action is

still unknown So far it has been found only in rabbit leukocytes and has not been detectable in cells from other animal species

A third principle and one most interesting from my personal standpoint is an anti anthrax substance' discovered by Bloom Watson, Cromartie and Freed (11) in areas of inflammation They characterized this substance as a strongly basic polypeptide or mixture of polypeptides with a lysine content of about 30 per cent These polypeptides probably originate from nuclear histones Indeed histones from various organs possess marked 'anthracidal properties

The bactericidal action of histones is not restricted to anthrax bacilli or spores Miller Abram, Dorfman and Klein in 1942 (12) described the bac-

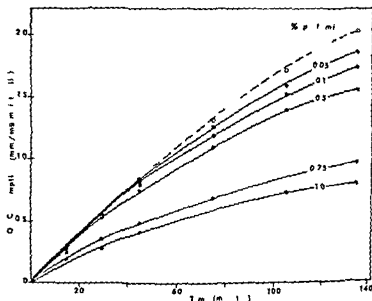


FIG 8 —Effect of protamine on respiration of tumor cells

tericidal activity of histones and protamines on a series of Gram negative and Gram positive micro organisms Unaware of these earlier observations Dr Brandis and I observed the bactericidal action of histones and protamines two years ago (13) We found that protamines and histones can be listed among the most potent bacteriostatic and bactericidal agents thus far known in addition they are able to inactivate or neutralize several types of typhus and coli bacteriophages Moreover, they are highly cytotoxic when incubated with all types of cells in a protein free medium This is illustrated in Figure 8 The respiration and glycolysis of cells incubated in a medium containing 0 1 per cent protamine or histone are effectively decreased

In this connection it is interesting to note that the group of polypeptide antibiotics, including tyrothricin, gramicidin, bacitracin the polymyxins and

viomycin are all more or less basic in character Although most effective in vitro some of them in common with the protamines and histones are toxic in tissues they also cause kidney damage following intravenous injection

It is tempting to speculate that histones and their breakdown products as released at the site of inflammation might be the antibiotics of our own body ' Thus far one can formulate as a working hypothesis only that their mode of action has something in common with the mode of action of some known polypeptide antibiotics

We have studied the effect of protamines and histones on various cells and with a view toward possible explanation of the above mentioned problem I should like to present our observations (14) When ascites tumor cells liver



FIG 9 —Liver cells of dog stained with hematoxylin eosin *a* Untreated *b* after treatment with 0 1 per cent protamine sulfate

cells or leukocytes are incubated with dilute (0 1 per cent or less) solutions of protamines or histones at pH 7 they immediately swell This can easily be observed with a phase contrast microscope (Fig 9) The staining properties of the treated cells are greatly altered the protoplasm has a greater affinity for acidic dyes (eosin) and nucleic acids inside the cell nucleus are no longer available for the attachment of basic dyes (methylene blue)

Boiled extract of protamine treated cells contains a considerable amount of basic protein which can easily be separated from other non coagulable proteins by means of paper electrophoresis After hydrolysis the basic protein has the same amino acid pattern as the original protamine From this observation we conclude that whole molecules (protamines having a molecular weight of about 5 000 and histones 20 000) permeate the still intact cell membrane and become more or less firmly attached to intracellular constituents

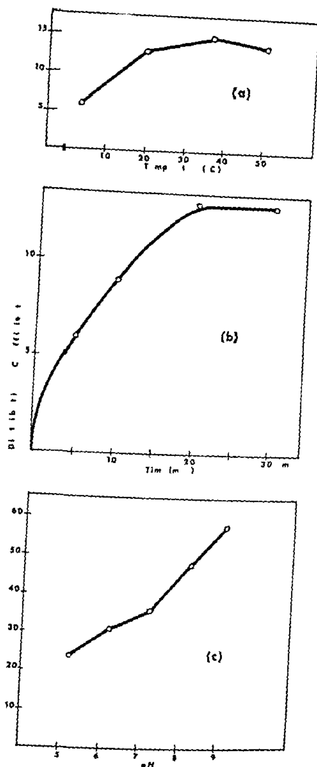


FIG 10.—Uptake of protamine by ascites tumor cells as a function of (*a*) temperature (*b*) time and (*c*) pH

Using ascites tumor cells, we have studied the kinetics of the uptake process. By incubating weighed amounts of cells with a given amount of protamine and under various conditions, the distribution coefficient was determined as expressed by the concentration ratio of intracellular to extracellular protamine. It turned out that the uptake is temperature dependent, reaches a maximum within 20 minutes at 32° C, and shows a typical adsorption isotherm (Fig. 10). By shifting the pH to the alkaline side, the uptake can be increased considerably. The process is almost completely irreversible, since repeated washings of the cells resulted in the release of only about 25 per cent of the protamine originally taken up. All data so far reported indicate that the uptake process is one of *passive adsorption*.

TABLE 2

INHIBITORY ACTION OF METABOLIC POISONS ON UPTAKE OF PROTAMINE BY ASCITES TUMOR CELLS

(Protamine Concentration 0.1 Per Cent)

Experiment	Poison	Distribution Coefficient	Inhibition (Per Cent)
II 21	None	25	
	Sodium cyanide 0.001 M	9	0
	Iodoacetate 0.001 M	12	53
II 24	None	61	
	Sodium arsenite 0.001 M	37	40
	Sodium fluoride 0.001 M	40	34
II 18	None	29	
	1,3 Dinitrophenol 0.001 M	9	0
	Sodium azide 0.005 M	20	30

In contrast to this, cyanide, fluoride, arsenite, dinitrophenol, and procaine inhibited the uptake to a certain extent (Table 2). These observations lead to the conclusion that some active transport mechanism might also be involved.

At first sight there appear to be two mutually exclusive alternatives: (1) passive adsorption and (2) active uptake. There is, however, a way to reconcile these findings. Three years ago I formulated a hypothesis (14) that cytoplasm should be regarded as an exchange resin which is kept in constant movement by metabolic forces. The material adsorbed at the surface is thus carried to the interior of the cell. When the movement of the resin is stopped by metabolic inhibitors or procaine, the transport of adsorbed material is consequently inhibited. At that time I did not feel bold enough to publish this idea. However, I am pleased to report that the underlying ideas, greatly supported by electron microscope findings of Palade, have been expressed as the *concept of membrane flow for active transport*. Figure 11 is a diagram taken from Bennett (15) illus

trating this concept It is based on the currently established fact that protoplasm in living cells is constantly moving the same applies to the cell membrane which might even have some contractile properties Molecules bound at first to the cell surface would be carried along the moving membrane deep into the interior of the cytoplasm Here according to Palade vesiculation occurs followed by dissolution of the vesicle with the result that the particle is inside the cell without having crossed the membrane This tempting concept would indeed explain the uptake of low molecular weight basic proteins as an adsorption process which at the same time can be inhibited to some extent by metabolic poisons

To return briefly to the mode of action common to basic polypeptides (as found in pus and in inflamed areas) and the polypeptide antibiotics I wish to

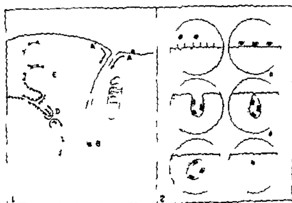


Fig 11 —Concept of transport by membrane flow See text (From Bennett (15))

refer to a paper by Newton (16) Using a fluorescent derivative of polymyxin (1 dimethyl aminonaphthalene 5 sulphonyl chloride coupled with the γ amino group of polymyxin) Newton was able to show that 90 per cent of the adsorbed fluorescent conjugate was associated with microsomes the small particle fraction and only 10 per cent with the cell walls

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CHAPTER 18

Some Cytochemical Studies with the Electron Microscope*

Isidore Gersh

THERE are two classes of cytochemical data in the literature of electron microscopy. The first comprises those dealing with the morphologic relations of calcium salts in bone, cartilage, and dentine, where the calcium salts are readily photographed with the electron microscope without any chemical treatment whatever. The specifically higher density of the calcium salts as compared with the protein structure serves to identify them, as they cause enough electron scattering to result in contrast on the photographic emulsion. The second kind of cytochemical data comprises those situations where certain substances are identified through the intermediation of some chemical process designed to cause increased mass at the site of these substances and hence greater electron scattering and a correspondingly increased contrast on the photographic emulsion. This category is treated in two papers which described the use of silver in attempting to identify DNA in sperm (5) and glycogen in liver cells (8), and two others devoted to the identification of alkaline and acid phosphatase (4, 9). In the last instance it was claimed that precipitated phosphate could be identified against a background of osmium used as the protoplasmic fixative. The underlying technical assumptions of these four papers seem to be too limiting. These are (1) that only heavy elements can cause enough electron scattering to enhance contrast differentially on a photographic plate in the electron microscope and (2) that fixation adequate to preserve cell structure sufficiently to withstand chemical reactions can be achieved only, or primarily, with heavy elements. In addition to these

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three other suggestive papers for cytochemical staining of submicroscopic structures have appeared (1 2 7)

I do not intend to discuss the question of artifact production either in frozen dried preparations or in preparations fixed by immersion in a buffered solution of osmium tetroxide My point of view for this chapter can be summarized in the following way After freezing and drying of different kinds of cells, protoplasm appears to have a certain basic structure Whether this closely represents the structure of protoplasm in the living cell or whether it represents a series of artifacts peculiar only to this method of preparation is for my purposes irrelevant Descriptive cytochemistry of any sort which involves dead cells or parts of cells no matter how they are fixed or even when they are not fixed, is based on this commonly understood assumption Cytochemical observations are always presented within a framework of a certain morphological substratum I shall first describe this morphological substratum which is observed after ultra rapid freezing and drying followed by suitable post fixation and staining, and then within this framework describe the results of applying a variety of cytochemical tests These were designed for the localization of glycogen basophilic substances (primarily RNA and DNA) certain enzymes and the highly diffusible ferrocyanide ion At least some of the cytochemical observations can be incorporated successfully into meaningful physiological concepts

Small pieces of tissue specimens are frozen rapidly by immersion in propane at about $-175°$ C The rate of freezing exceeds 5 000° C sec and the specimen is frozen in about $\frac{1}{50}$ second (10) The specimens are dried at about $-35°$ C Such specimens may be post fixed and stained in a variety of ways and the images photographed with the electron microscope constitute the morphologic substrate for the cytochemical procedures I should like to show some of the main features of the structure of liver cells as they appear after freezing and drying followed by staining with platinum tetrabromide (Figs 1 and 2) lead acetate and uranium nitrate (6) In both the nucleus and the cytoplasm the ground substance appears as a series of discontinuous submicroscopic vacuoles about 500–900 Å in diameter imbedded in a denser continuous matrix which constitutes the walls of the vacuoles The walls may be 100 300 Å thick The submicroscopic vacuoles and their walls are interpreted as a two phase system—the submicroscopic vacuoles consisting of a less dense water rich protein poor phase their walls consisting of a more dense water poor, protein rich phase Both phases are regarded as being in dynamic equilibrium and subject to change in difference states of activity Mitochondria appear homogeneous with most stains though with some stains they contain small rodlets and sometimes minute vacuoles or granules I have not seen membranes in or on the mitochondria around the nucleus or in or around the

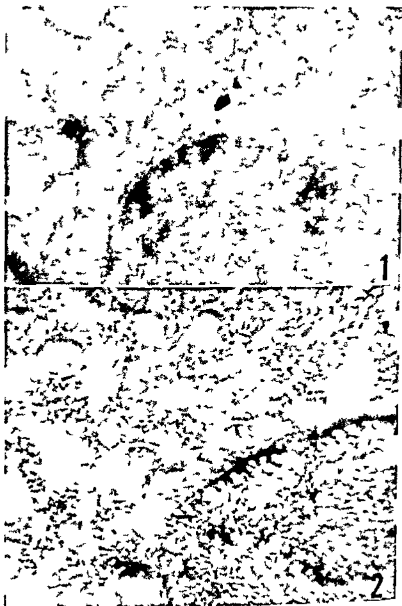


Fig 1 —Portion of a liver cell of a guinea pig prepared by ultra rapid freezing and drying after post fixation and staining with platinum tetrabromide The two phase vacuolar system is present in both nucleus and cytoplasm The walls of the submicroscopic vacuoles (the denser phase) vary in thickness and density The mitochondria are uniformly dense ×16 000

Fig 2 —Portion of a liver cell of a guinea pig prepared as in Fig 1 Some of the mitochondria are vacuolated ×17 000

cytoplasm When the specimens are frozen less rapidly ice crystals appear and these are recognized as areas of minimal electron scattering with denser walls (Fig 3)

There are many who are concerned with membrane properties in the transport of substances in and out of cells and within cells and who are dismayed by the absence of lines in the photographic image representing membranes I would remind such individuals that the essential requirement for membrane function is an interface and not a line The specific properties of such an interface would depend on the composition enzymatic and otherwise of the materials on *both* sides of the interface I would suggest that such interfaces are



Fig 3—Portion of a liver cell of a guinea pig with ice crystal The ice crystals formed probably because the liver specimen was not frozen rapidly enough The ice crystals appear clear and are larger in the nucleus (N) than in the cytoplasm The larger dark masses in the cytoplasm are mitochondria (M) which have been distorted by the growth of the ice crystals ×10 000

provided by the submicroscopic vacuoles and their walls In the nucleus of the liver cell it is estimated that there are 500 000 such interfaces as that is the number of submicroscopic vacuoles estimated In the cytoplasm the number runs into the millions Looked at from this point of view with the method of preparation described earlier more interfaces can be seen than by any other method I suggested previously that the submicroscopic vacuoles and their walls are in a state of dynamic equilibrium and are hence subject to constant change Each such change in time thus increases even more the total number of interfaces beyond that which could be counted or estimated at any one moment

At the interface between the nucleus and the cytoplasm what seems at low magnification (with the light or electron microscope) to be a "membrane is resolved at higher magnification into submicroscopic vacuoles and their walls It is hardly necessary to point out that the composition of the walls on both the nuclear and the cytoplasmic sides differs markedly at least in respect to protamine, histone, DNA RNA and a variety of enzymes Thus there is every reason to expect that, at the critical interface between nucleus and cytoplasm, one should expect differences to occur in the permeability and transport properties of the "nuclear membrane'

Against this kind of background of submicroscopic morphology, I should like now to show you some pictures of glycogen in liver cells that were made by Bondareff (3) The glycogen was stained specifically by the periodate leucofuchsin reaction The dye consists of C, H, N, and O and has a molecular weight of 324–37 Yet the increase in mass due to the added dye was sufficient to increase the density of the glycogen to the point where it caused a differentially enhanced electron scattering adequate to identify the glycogen dye complex selectively with the electron microscope At the level of the light *microscope, glycogen appears in the form of granules or rodlets* 1–2 μ (Fig 4) If an equivalent specimen is first treated with saliva to digest the glycogen, there is no staining, or it is barely appreciable (Fig 5) With the low power of the electron microscope, the granules are again selectively stained (Fig 6) and again no clear granules appear in the salivary digestion control (Fig 7) At higher powers, these granules are resolvable into smaller units of about 600–900 A (Fig 8), and at a still higher power these are, in turn, resolvable into small granules of about 100 A (Fig 9) With the highest resolution and magnification, something can be seen of the shape of the finest particles *There* is reason to believe that the 600–900 A particles are in submicroscopic vacuoles and that a cluster of such particles comprises the granule visible in the light microscope There is reason to believe that the smallest particles may be the denser core of the glycogen molecule

The chief structures which stain with basic dyes are the chromatin and nucleolus of the nucleus and the ergastoplasm or chromidial substance of the cytoplasm One reason for relating this staining property to the occurrence of DNA and RNA in these structures is the effect of DNAase and RNAase on the staining properties Treatment with DNAase abolishes or greatly reduces the basophilic staining of the chromatin and chromosomes Similarly treatment with RNAase abolishes or greatly reduces the basophilic staining of the nucleolus and the ergastoplasm I should like to show you some of the work of Finck with the basic dye gallocyanine chromalum on liver cells and of Isenberg with the same dye on salivary gland chromosomes of *Drosophila* In the cytoplasm of liver cells, basophilia appears in some small granules of about 100 A or more These are located primarily in the walls of the submicroscopic



5



7

FIG 4.—Glycogen granules in liver cells of a guinea pig. Prepared by ultra rapid freezing and drying with post fixation in alcohol and staining by the periodate leucofuchsin method. Section 4 μ thick photographed with an oil immersion objective ×2,100

FIG 5.—Specimen from the liver of the same guinea pig prepared by ultra rapid freezing and drying with post fixation in alcohol. The specimen was then treated with diluted saliva to remove glycogen and then stained, sectioned and photographed as in Fig 4. This is a control preparation ×2,00

FIG 6.—Glycogen granules stained specifically as in Fig 4, imbedded in methacrylate and photographed with the electron microscope ×4,000

FIG 7.—Control preparation for Fig 6. The specimen was treated as in Fig 5, imbedded in methacrylate and photographed with the electron microscope ×4,000

8

9

FIG 8 —Glycogen granules prepared as in Fig 6 at higher magnification to show that the granules visible with the light microscope are resolvable into smaller particles of about 600–900 Å ×18 500

FIG 9 —Glycogen granules prepared as in Figs 6 and 8 at still higher magnification The intermediate sized granules contain the finest resolvable units of about 100 Å which are believed to be the denser core of the glycogen molecule ×63 000

vacuoles Pretreatment with RNAase abolishes this staining. In the nucleolus basophilia occurs as granules of about 80 A and this selective staining is also abolished by RNAase In the chromatin basophilia occurs also as granules in the thickened walls of submicroscopic vacuoles and this selective staining persists after RNAase but is abolished by DNAase Some idea of the specific contrast introduced by the stain may be gained by comparison with an unstained specimen (Figs 10 and 11 12 and 13) Chromalum alone which is one of the components of the stain does not improve contrast The gallocyanine chromalum lake alone is effective In this instance increased density of the basophilic components is achieved by C H N and O together with the inorganic components of the lake with altogether a molecular weight of about 400 One great advantage of this stain over others is that it is self terminating that is it stains only the basophilic components without over staining

I turn now to the salivary gland nuclei of *Drosophila* After staining with gallocyanine chromalum the basophilia appears in the form of small granules occupying the walls of the submicroscopic vacuoles in the chromatic bands of the chromosomes and somewhat more diffusely in the nucleolus (Figs 14 and 17) Prior treatment with RNAase greatly reduces the electron density of the nucleolus (Figs 15 and 18) while DNAase virtually abolishes the contrast of the dark bands (Fig 16)

I should like to call attention now to another set of problems involving the localization of enzymes and of SH groups This work is now being done by Dr Nelson Rat epididymal fluid is frozen and dried After brief rewetting of the specimen in a suitable buffer the substrate is added and after about 3–30 minutes the reaction is stopped The specimens are imbedded sectioned and viewed The accumulated reaction product or the addition product with a suitable inhibitor increases the mass locally and this increases the electron scattering correspondingly which again causes enhanced contrast in the same sites The situation is summarized briefly in tabular form (Table 1)

The morphological basis for the studies appears in photographs of sperm tails stained with *platinum tetrabromide* Of more immediate interest are the peripheral fibers inclosed in a sheath containing a helical coil ATPase appears to be most active in the longitudinal fibers (Figs 19–22) Alkaline phosphatase and succinic dehydrogenase (Figs 23 and 24) appear to be most active in the helical coil and in the fibers respectively Sulfhydryl appears in these sites also as well as in the matrix

The ATPase is regarded as an energy source and is presumably associated with the contractile protein Its richness in the fibril lends support to Bradfield s suggestion that the fibrils contain contractile elements The close association with succinic dehydrogenase might be expected from the fact



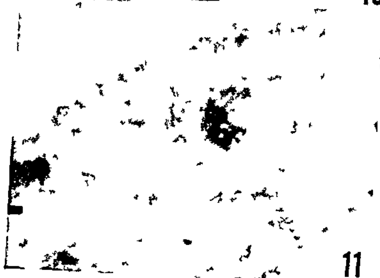
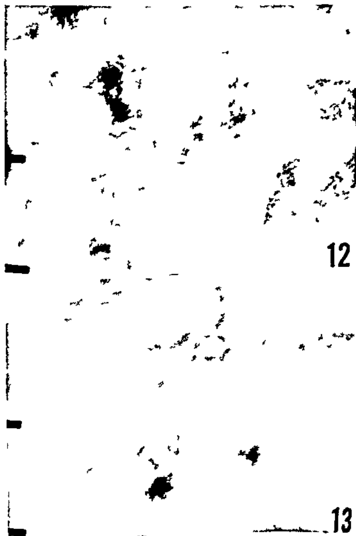
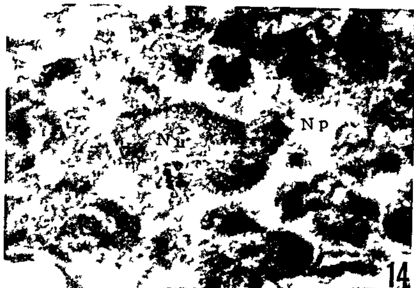


Fig 10 —Nucleus of a liver cell of a rat prepared by ultra rapid freezing and drying heat and alcohol post fixation and staining with gallocyanin-chromalum The selective nuclear staining is confined to the chromatin bodies As compared with Fig 11 the chromatin shows an enhanced contrast ×13 300

Fig 11 —Nucleus of a liver cell of a rat prepared as in Fig 10 but not stained with the dye ×13 300



12 —P rtion of a nucleus of a l ver cell stained as in Fig 10 to sho s that tl
r j hilia is c nfine l largely t granules wh ch are part f the chromatin
13 —Port n of a nucl us of a li er c ll prep re l with ut staining as in Fig
are lark l ut the gra les of l asophil c m terial in them are n t appar
ne l ×2(500



C

Nu

15

16

FIG 14 —Part of a nucleus of a salivary gland cell of *Drosophila* prepared by ultra rapid freezing and drying post fixation by heat and alcohol and staining with gallocyanin-chromalum The nucleolus is the dark unbanded body near the center of the photograph (*Nu*) The remainder of the deeply staining bodies are parts of chromosomes (*C*) some of which show sharp bands The nucleoplasm (*Np*) is the paler material between the chromosomes and between them and the nucleolus All components show the two phase structure ×7 000

FIG 15 —Part of a nucleus of a salivary gland cell prepared by ultra rapid freezing and drying post fixation by heat and alcohol and digestion by ribonuclease followed by staining with gallocyanin chromalum The effect of the enzyme is to remove ribonucleic acid without greatly affecting the desoxyribonucleic acid As compared with Fig 14 the nucleolus (*Nu*) is much paler the interband regions perhaps paler while the granules in the dark bands of the chromosomes (*C*) are largely unaffected The enzyme causes some distortion and displacement ×7 000

FIG 16 —Part of a nucleus of a salivary gland cell prepared as in Fig 15 except that desoxyribonuclease was used (instead of ribonuclease as in Fig 15) to remove desoxyribonucleic acid The staining of the nucleolus (*Nu*) and the consequently enhanced contrast persists while the barest ... The enzyme causes some distortion and displacement ×7 000

17

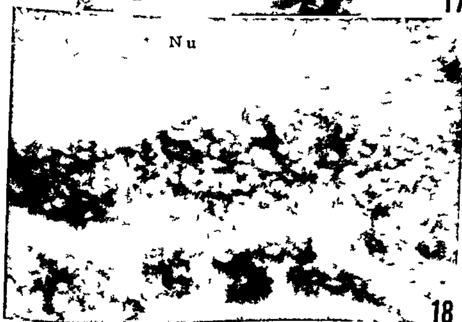


FIG 17—High r p we picture of a part f the ucle l s (Nu) a l perinucleolar el ments f Fig 14 t sh w the granules f the chrom s m ban l (C) ×21 000

FIG 18—H gh p we pictur f a part of the n cl ol r (Nu) and peri ucler la el ments of Fig 15 t show th granule of the chromos me (C) t n l pers t g aft r treat t w th ribonuclease The nucle lus has lo t m ch f co tra t a comp red with the specim n ot tr te l with this enzyme (Fig 17) ×21 000

that it is important in oxidative phosphorylation of ADP and thus the replenishment of ATP The sulfhydryls would be expected to occur widely, as they are essential components of various enzymes including ATPase

My last example of cytochemistry with the electron microscope concerns some preliminary findings of Dr Chase on the localization of ferrocyanide In these experiments ferrocyanide is injected intravenously into a mouse in a 20 per cent solution, and after a few minutes the mouse is bled The diaphragm is frozen rapidly and then dried Paraffin sections are made and the ferrocyanide is precipitated as Prussian blue with minimal diffusion The sections now containing the insoluble ferric ferrocyanide are imbedded in methacrylate and sectioned again for the electron microscope

TABLE 1

Enzyme or Reactive Group	Substrate	Reactant	Insoluble Complex or Reaction Product	Mol Weight of Added Substrate or Reaction Product
ATPase	ATP	Ca^{++}	Calcium phosphate	310 or less
Alkaline phosphatase	Glycerophosphate	Ca^{++}	Calcium phosphate	310 or less
Succinic dehydrogenase	Succinate	Polymeric tetrazolium	Formazan	300 or multiple
		Malonate	Enzyme inhibitor complex	148
—SH		N ethyl maleimide	SH inhibitor	175
		PCMB	Mercaptide	378

With the light microscope ferrocyanide appears in the form of discrete droplets of about 1–2 μ confined to the connective tissue between the muscle fibers (Figs 25–27) These are further resolvable as clusters of droplets of about $\frac{1}{4}$ μ (Fig 28) With the electron microscope these are still further resolvable into clusters of still smaller vacuoles of about 600 A or more (Fig 29) At higher magnification the ferric ferrocyanide appears to be in granular form There are some indications that the granules appear in the electron microscope as an artifact associated with a high electron beam intensity, but more pictures taken at a low beam intensity are required before we can be sure In the electron microscope, ferrocyanide becomes almost differentially visible because of its high electron scattering capacity since its molecular weight is 859

The segregated distribution of most of the ferrocyanide in the connective tissue came as a surprise, for we expected the very soluble ferrocyanide to be uniformly distributed in the water of the ground substance What we do not yet know is whether the ferrocyanide droplets correspond with a pre formed structure of the ground substance Nor do we know whether this segregation of

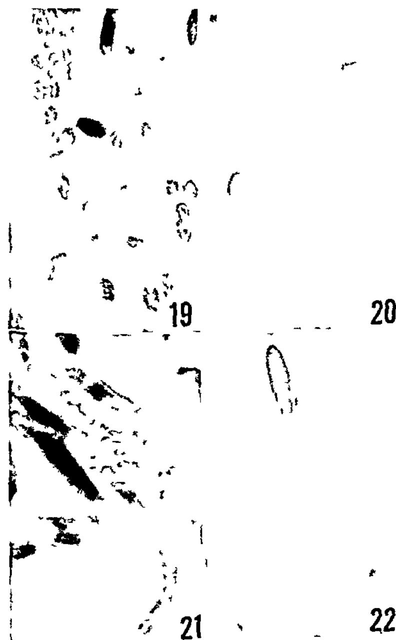


FIG 19 —Sections of sperm prepared by ultra rapid freezing and drying and immersed for 5 minutes in a buffer solution containing ATP As compared with the controls (Fig 20) there is enhanced contrast in the filaments due to the deposition of a calcium phosphate compound derived from calcium ions in the buffer and phosphate ions liberated from the ATP The darkest bodies are sections of sperm heads ×10 000

FIG 20 —Control specimens prepared as in Fig 19 except that the buffer solution contained no ATP The dark bodies are sections of sperm heads ×10 000

FIG 21 —Section prepared as in Fig 19 showing enhanced contrast particularly in the longitudinally sectioned filaments

FIG 22 —Control specimen prepared as in Fig 20

the ferrocyanide may serve as an indicator of the existence in the ground substance of connective tissue of pools or sites where water is relatively abundant where positively charged groups are aggregated or where negatively charged groups are less dense

In conclusion I should like to make some general remarks about cytochemistry at the submicroscopic level In general, cytochemistry with the electron microscope suffers from all the defects of cytochemistry with the light micro-



FIG 23 —Sections of sperm prepared by ultra rapid freezing and drying and immersed for 10 minutes in a succinate buffer solution containing malonate As compared with the control (Fig 24) there is enhanced contrast in the filaments and the outer pellicle of the sperm tail This is attributed to formation of a complex of malonate with succinic dehydrogenase In later work the enhanced contrast was more restricted to the filaments ×10 000

FIG 24 —Control specimens prepared as in Fig 23 except that the succinate buffer solution contained no malonate ×10 000

scope and in addition some others With the light microscope specificity sensitivity diffusion and fixation are the major problems With the electron microscope in addition to these are others which enter from the lack of color and the more stringent technical requirements associated with increased resolution the greatest of these being the retention of the fine structure of protoplasm despite the vigorous chemical and physical treatment incurred during the cytochemical manipulations Ideally every cytochemical procedure requires that the substance tested for be treated as if it were in solution yet it must remain insoluble and not shift more than a few Å units On the other hand

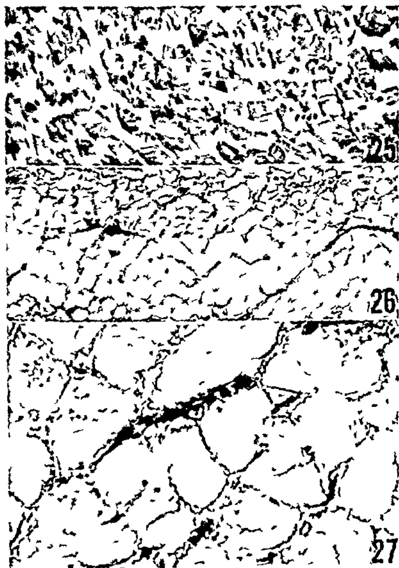


FIG. 25.—Ph ɪt mɪ rograph f a nea h trans er e sect on thr ugh a mo e d ıɑ hragm ɪ rej ar f ɪ ɪ ultra raɪ ıd freez ɪ g and *drying* amt e f ling n ɪ ara f n an l taın ng ɪth h *n* at xɪ l n an l eosin. *The* nuclei are staine l d ɪ ɪ ly and the rema n l *of the ɪ* rot ɪ lasm less ıntensely ×2 0

FIG. 26. I h t mı graɪ h *of a* secti n f the same dıaɪ h agm a h *r in* F g 23 l ut ta ned f r ferrocyanide only The nucleus an l cyt ɪ la m f all cells ar un ɪ ned The f rocy n le appears *blue in the* ecti n a d l lack in the ɪ hotograɪ hs Ferroc anı le f n f enti lɪ ɪ t e connective tissue between the muscl f l ers ın l iscret regions ×2 0

FIG. 27.—Higher pc er ɪ h tomicrograɪ h *of a* ɪ n la ɪ r ɪ rat n to h some clu ters f ferrocyanide ×1 000

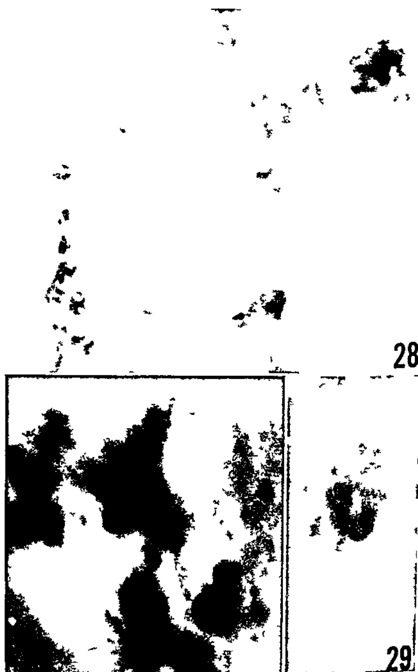


FIG 28.—Further enlargement of some of the clusters to show that the ferrocyanide clusters are composed of still smaller aggregates of about 0.3 μ confined, as above, to the connective tissue ×3 000

FIG 29.—Further enlargement of some of the clusters The smallest ferrocyanide aggregates observed with the light microscope comprise groups of submicroscopic vacuoles of about 600 Å or larger in diameter ×40 000

a simpler situation obtains in working with the electron microscope in certain situations as for example, in the test for ATPase and alkaline phosphatase where successive treatments with aqueous reagents to induce color are avoided and in the test for —SH and succinic dehydrogenase which become photographable because of the increased mass caused by the relatively simple additions or other type of complex formation

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CHAPTER 19

Structure and Function in Nerve*

Julian M Tobias and Phillip G Nelson

THE specific area with which this chapter is concerned was last reviewed in 1952 (88 89) Since then many papers have appeared which deal with nerve Some have been concerned with functional matters others with morphology but only a few report experiments designed explicitly to yield information on structure function interaction that is to say on the functional expression of structural state Thus although much has appeared concerning such matters as electron microscopy or X ray diffraction studies of neural and related elements the meaning for function is almost exclusively implicit Only rarely is explicit evidence of functional status obtained simultaneously with the morphological information The high resolution techniques those which are potentially able to tell most about protoplasmic or cell constituent organization at the molecular level are still esoteric enough that only relatively few attempts have been made to use them as tools among tools They are for the most part used for what they can reveal independently and combining them with other techniques such as those commonly used in investigations of excitation is only beginning It requires of course either collaboration among workers trained in the several areas or individuals willing to work—and perhaps err in both In any case this coming development has about it an aura of high excitement One feels that there will soon come a shift from almost exclusive emphasis either on electro ionics or submicroscopic morphology to a newer emphasis on integrating the two

FORMULATION OF THE PROBLEM

In an interval of about 1 millisecond a segment of axon can go from a resting state, through a transition phase to a state of maximal excitation and back again Accompanying these events there have been thought to be changes

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in oxygen consumption carbon dioxide production heat production ammonia production and creatine phosphate degradation but these events have never been proved to accompany the phase of generation of activity that period when changes occur which then permit trans surface ion movements to express themselves as the action current and action potential Indeed they are known long to outlast it and to be associated in large part, with rather slow recovery events Thus it has been known for many years that although nerve gradually fails in nitrogen (26) or carbon monoxide (79) the latter being reversible by light, such inexcitability is complete only after some hours More recently it has been shown that the increased oxygen consumption which normally accompanies activity in nerve can be suppressed with sodium azide without interfering with impulse conduction (6) and axons can continue to transmit many impulses even after being treated with dinitrophenol azide or cyanide to the extent of preventing extrusion of the sodium which has entered during activity and reaccumulation of the potassium which has left (45) Similarly it is now known that phosphate esters of the squid axon can be reduced to low levels without interfering with the ability to conduct action potentials (10) Thus nerve impulse generation per se and the immediate recovery process may both be essentially ametabolic Perhaps they are best thought of as an expression of a kind of transient death of the cell during which time the system built up by metabolism during the resting state is allowed for an instant to run down Electrokinetic and structural bases for such a phenomenon have been briefly speculated upon elsewhere (88 91)

In the resting state the axon accumulates potassium maintains intracellular sodium at a low level and exhibits relatively high trans surface capacity and impedance i e ions do not freely move in and out they are prevented from running down their concentration gradients The resting state is also characterized by a trans surface potential of *ca* 50–100 mv poised so that the cell is negative inside relative to the extracellular environment

Electrically when the axon starts to go toward the active state it first exhibits symptoms which reflect its construction as a core conductor i e it responds to the impressed charge the stimulus in a fashion described by the core conductor equations However it soon adds to this so called passive behavior and changes then appear which are due to other than core conductor properties it begins to respond to the impressed charge in a non linear fashion This non linearity increases until suddenly as if a trigger had finally been pulled past a point of no return the change becomes explosive and the state of maximal excitement is rapidly reached

A fundamental feature of this behavior is the progressive change of the trans surface potential from its resting level of 50–100 mv with the cell interior negative through a zero level to a point when the cell interior is 30 or so

my positive Recovery is characterized by a reversal of this state of affairs and by re establishment of the rest potential and poise

This cycle of changes in potential is an expression of transfer of charge In the earliest stage, described as the period of core conductor behavior it is *as if the charge is being neutralized on a condenser at the axonal surface* This period and a brief interval thereafter constitute the time of triggering Then *transfer of charge begins to occur in a grosser fashion the flow of capacity current* is replaced by trans surface ion movements, with sodium ions apparently entering the cell and potassium ions leaving it (39–43) It is these ion movements which, *for any microlocus on the axon are the so called action current The voltage changes accompanying their movements are the action potential*

Now then, since a major matter in the change from the resting to the active state is this transfer of charged particles, a movement of ions across the cell surface, or at least across some part of the cell surface the questions arise *Why do these movements take place so relatively freely at one time the period of activity but only to a limited extent during the resting state? What in molecular structural terms happens during the triggering period which then permits these freer ion movements? How is this process limited, stopped and reversed? These are some of the most interesting questions being asked about excitable cells and probably they apply in some way to all cells*

Our laboratory has adopted a dual approach to the problem On the one hand, it has been thought that if a change in molecular level structure is what *permits the ions to move during activity, then perhaps such an event would be detectable as a change in mechanical properties of the nerve occurring as* an accompaniment of activity It has been hoped that such changes, if detectable *might be amenable to further analysis finally yielding up the names of* participating molecules As an opposite approach attempts have been made to modify the structure in a chemically known way, as *for instance, by the* application of enzymes to the living system, these structural alterations then being monitored for associated changes in axonal functional competence and *for some visible changes in morphology The first approach asks* "What structural changes occur with activity and what underlies them? The second asks, "*What functional changes occur when the structure is modified, and how do they relate to that structural modification?*

The following will summarize contributions to our information about these matters which have been made since a review (89) was written by *one of us* in 1952 as well as some material of which the writer of that review was *not* aware at the time Since most of the electron microscopic morphologic studies *in this area have been made without functional correlation* it is difficult to relate them to the activity except in a purely speculative way They are greatly interesting and most important however, and, as will be seen they raise inter

esting questions hence they will be considered with certain other as yet unco ordinated observations

SUBMICROSCOPIC MORPHOLOGY AND IMPLICATIONS (EXCLUSIVE OF CELL BODY)

Contributions have been made by two major techniques—X ray diffraction and electron microscopy In the former category most of the work has had to do with the structure of frog nerve myelin and is summarized in a recent paper by Finean (20) In the radial direction the fundamental repeating unit in myelin is thought to consist of a 30 A layer of protein associated with a bimolecular lipid layer about 55 A thick As suggested earlier by Winkler and Bungenberg de Jong (97) the lipid layer is also thought to contain cholesterol, so positioned that when it is hydrogen bonded to the NH of cephalin or sphingomyelin it helps to bring the phosphate part of the phospholipid closer to the underlying protein

This general schema modified to apply to lobster nerve which requires taking into account a preponderance of lecithin over cephalin (*ca* 5 1) and a preponderance of total phospholipid over cholesterol (*ca* 2 1) (5) and which contains just about as many calcium positive charges as phospholipid negative charges has been used in an attempt to explain certain functional and electron microscopically demonstrable changes produced in lobster axons by the application of proteases or phospholipases (91)

The only experimental attempts known to the writers to correlate X ray data with functional state are those of Handovsky (32) and of Boehm (3) These workers have observed variable changes in pattern in the electrically stimulated or strychninized frog spinal cord and in the spinal cord exposed to chloroform Although the change produced by chloroform was inconstant it appears to have been real It was concluded that the effects were somehow due to changes in organization of the *Fettsauremolekule* Elkes and Finean (15) have found little change in the X ray diffraction pattern of fresh frog nerve in the range above freezing until reaching 58 -61 C In this context Kayushin *et al* (49) call attention to the fact that failure of excitability with rising temperature occurs in the frog nerve at about 35° well below the temperature at which changes occurred in myelin X ray diffraction It was therefore concluded that rising temperature can inactivate the excitable system by some means which does not modify myelin structure

Electron microscopic studies have been fruitful as regards both myelinated and so called unmyelinated nerve The lamellar structure of myelin established by X ray diffraction has been amply confirmed (21) and in addition Geren (28) contributing importantly to the problem of the mechanism of myelinogenesis has obtained evidence that myelin is somehow deposited around axons in a spiral fashion by Schwann cells Another somewhat different example

of the same fundamental process, i e, inclosure of axons in double lamellae inside Schwann cells is seen in a picture of small fibers shown in such an arrangement attached to the encompassing Schwann cell surface by a so called mesaxon (25)

A question of great interest to many physiologists is the following Does a chemically or structurally unique metastable single membrane about 30–100 A thick—whose state at the molecular level is thought by many to determine whether the axon is at rest or excited or indeed to be synonymous with the resting or excited condition—exist as a physically discernible entity distinct on the one side from axoplasm and on the other, from myelin? The tacit assumption of an affirmative answer to this question has colored much experimental and theoretical work apropos the various aspects of excitation However even though electron microscopy can resolve structures of such dimensions the fact is that this technique has not yet given an unequivocal answer to the question Several reasons suggest themselves First of all, a crucial parameter in the definition of this structure and therefore a crucial feature for its recognition has been the assumption that it is unstable and somehow changes with the rest excitation recovery cycle Since a part of these changes at least will endure only for times of the order of milliseconds or less and will probably involve only part of the surface ordinary fixation and electron microscopic methods cannot capture them in any simple way Second, although certain patterns seem well preserved by osmium (19) there is the possibility that fixation, imbedding etc might destroy such a structure before it could ever be demonstrated electron microscopically even if were it preserved it would somehow intuitively look like *the* membrane Third as noted earlier only little effort has been made to use electron microscopy as a tool in functional studies (91)

In spite of this however what pertinent facts are available from electron microscopy? To begin with the axon has a limiting surface before myelination Since this surface can still be seen while myelin is being deposited (28) and since the axoplasm at the node has a distinct surface layer where all (other) myelin has been interrupted (66) and since at the synapse there appear to be only the axonal and cell body surface layers with no intervening myelin there would seem to be no question that there is some sort of condensed structure at the axoplasmic surface independent of later deposited myelin Is this layer however either structurally or chemically different from the later deposited myelin? On the basis of early work on the internodium of myelinated fibers, some investigators believed that they had evidence of a unique axoplasmic surface layer different from more peripheral myelin (2, 17 34, 71), while others concluded that such a layer was not demonstrable (78), and still others were unsure (75) As techniques improved one of the first group was led to change his mind toward the view that such a layer is not demonstrable (18)

In this laboratory no clear evidence for a unique surface layer in the internodium has been seen Therefore perhaps one can conclude that on the basis of such electron microscopic studies a unique membrane as defined above does not exist in the internodium However in myelinated nerve fibers excitation apparently takes place only at the node (84) Therefore perhaps one should search for such a unique layer only at the node and not in the internodium Since the nodal area is described in detail below suffice it to say here that the axoplasmic surface layer there also has not appeared to be unique

For unmyelinated fibers the information is somewhat more satisfactory but perhaps only because fewer investigators have worked on them Thus from work of Geren and Schmitt (29) and of Tobias (91) it is seen that both squid and lobster axons exhibit a multilaminated surface structure made up of a series of tangentially oriented radially connected osmiophilic double layers each with a less densely osmiophilic interval these layers being interpreted by Geren and Schmitt as part of the Schwann cell Whatever their origin wherever two or more such triple units are present the one most intimately applied to the axoplasm does not look different from some others farther out No unique axoplasmic surface layer has yet been demonstrated As will be discussed later certain enzymes have effects on the structure of these layers which when examined in the light of the concomitant functional competence of the axon allow some interesting speculation about their more detailed composition (91)

To summarize electron microscopy has not yet been used in a way which can be accepted as having demonstrated *the* membrane to be an entity either structurally or chemically different from myelin lamellae as ordinarily recognized On the contrary to the extent that the data can be accepted at face value any condensed layer seen at the axoplasmic surface so far does not appear different from similar more peripheral ones However there is one sense in which the innermost layer is unique and this uniqueness is due to its position It is the only layer in contact with axoplasm on one side and non axoplasm on the other Since the chemical and physical properties of interfacial structures are conferred on them largely by the nature of the contacting phases this innermost layer may be peculiar not because it is made of stuff different from that in more peripheral layers but because its components are exposed to two different and peculiar bulk phases In the same sense the outermost layer with Schwann cytoplasm on one side and extracellular fluid on the other may be unique Thus these layers unique because of place may have properties of importance mainly because position makes their composition biologically significant

To the extent that the results do describe the axon surface one is forced to consider new dimensions in the problems of trans surface exchange of ma

terials For example Are there mechanisms underlying the movement of ions and other substances across multiple layers, as at the surfaces of lobster or squid axons which are qualitatively different from those operating at the nodal area? Are there structural discontinuities in these layers (91)? Is a pinching off of invaginated segments of the innermost Schwann cell layer a device for transporting certain materials (mitochondria?) into the axoplasm (29)? How do lipid and protein components of the lamellae interact to confer spatial orientation on each other (91)? Of what importance is such spatial orientation for metabolism? What sort of bonds in such a structure might be most sensitive to change by current (91)? These are the sort of considerations that were referred to in the introduction as presenting new problems of structure function interaction

Nodal structure has not yet been satisfactorily displayed in the literature although some electron micrograms have appeared (24, 34 70 71)[1] The impression gained by Hess and Young (33) that the myelin demonstrable by light microscopy is completely interrupted at the node has been confirmed by electron microscopy (66) However, this region requires description as the node is approached the myelin lamellae turn in apparently to terminate at a surface layer on the axoplasm This termination is characterized, in some instances at least by a small bulbous expansion of the turned in end of the myelin lamella The innermost axoplasmically applied layer which these lamellar endings seem to contact extends on to bridge the nodal gap As discussed above this innermost layer has not yet been demonstrated to be structurally or chemically different from the lamellae which are obviously myelin The perinodal space is populated by a variety of small bodies with internal structure The axoplasm contains vesicle like structures which may be more concentrated at the nodal area and are reminiscent of similar pre synaptic particles (see below) A comparable description of the nodal area has been published by Robertson (77)

Interesting issues are also developing because of advances in the electron microscopy of junctional regions (16 68 69 73–76) Neuromyal and synaptic junctions in general seem to be characterized by a multilayered membrane like structure usually two (exceptionally as in the case of the chameleon neuromyal junction, five) layers thick separating pre from post junctional cytoplasm by a gap of several hundred A and approximated in the axoplasm of the pre junctional side by numerous vesicular or tubular structures In addition beginning a correlation with functional matters De Robertis (74) has shown the so called vesicles of retinal synapses in the rabbit to be smaller after light deprivation and to disappear in the acoustic ganglion cells of the guinea pig

1 After this paper was written the greatly superior electron micrograms of Uzman and Nogueira Graf (95) appeared

after cochlear destruction Perhaps a chemical transmitter of excitation (for general reviews see Del Castillo and Katz [11] and Eccles [14]) will be found to be associated with such pre junctional vesicles The recent findings of Bullock and Hagiwara (9) and of Tasaki and Hagiwara (85) further emphasizing the dominant role of a chemical transmitter at the giant axon synapse of the squid stellate ganglion are of interest in this connection

MECHANICAL AND LIGHT SCATTERING EXPERIMENTS

Resting Nerve

Relatively few observations have appeared which are pertinent to the structure of resting axons as revealed by thermal mechanical light scattering etc properties Dodt (13) measured the thermal co efficient of linear expansion of frog nerve and found it always to be positive over a range of 2 –61 C with a magnitude of about 10^{-4} per degree for intact nerve Dissection of the peri and epineurium reduced the magnitude of the coefficient to 10^{-5} per degree and this latter figure was considered to apply to the axons per se since treatment with collagenase failed to reduce it further The fact that the coefficient was positive as found also by Kayushin *et al* (49) for the physiological range is interesting because it may reflect a decrease in cohesion of structural elements with temperature rise and this may in turn be related to the positive temperature coefficient for electrical activity in nerve (23) though the magnitude of the temperature coefficient makes a purely physical explanation somewhat unlikely It was also reported by Dodt (13) that the length changes with temperature were fully reversible over the range 17°–32 C Kayushin *et al* (49) however found hysteresis effects even between 12° and 30 C and have also reported that with or without the sheath at constant length the long axis tension of the nerve decreases with temperature to about 30° C but then begins to increase At about this point of reversal excitability and impulse conduction fail Since as mentioned earlier X ray diffraction shows no change in myelin pattern below about 60 it has been concluded that the failure of excitability and the shift in mechanical properties at 30°–35° both reflect a change in some non myelin component which is important for excitability

Boyarsky (4) has measured Young s modulus for the isolated squid axon and finds it to be about 10^3 dynes/cm i e about 1/100–1/1 000 of the value for many solids His data are of interest primarily in terms of the stretching experiments discussed below

Kao (46) has described chains of oval vesicles about 30 × 50–150 μ in size in the superficial region of the squid axon axoplasm which are said to form from nodose swellings on filamentous structures visible a few minutes after dissection The vesicles are structureless but are apparently invested by a

thin membrane Keeping the axon in sea water or injecting Ca Mg, Sr, or Ba salts causes them to round up This is further evidence that divalent cations injected into the axoplasm cause it either truly to disperse or to undergo a pseudo dispersion by becoming agglomerated into very small particles Presumably this could be resolved by dark field microscopy

Lubinska (59 61) following the observation that in myelinated fibers the proximal juxtanodal bulb is always larger than the distal one considers this analogous to the axonal swelling shown by Weiss (96) to occur on the proximal side of a cuff applied in such a way as to constrict nerve *in situ* In an attempt to gain further insight into the mechanism of proximo distal flow in axons which presumably is responsible for both these effects Lubinska has made further observations on axonal distensibility and elasticity (58) and on the physical state of axoplasm (60) It has been found as shown earlier by Haig (22) for unmyelinated axons that myelinated fiber axoplasm is under tension and will be extruded from a cut end even when the axon is separated from the perikaryon (60) This force therefore may also play a part in the phenomenon of proximo distal flow It would be of great interest to know how much of such tension is due to osmotic forces and what sort of bonds in the structure are responsible for the surface elastic distensibility Such information might bear on the probableness of a structure proposed elsewhere for part of the axonal surface (91)

Active Nerve

That myelin confers appreciable mechanical support on the excitable system is suggested by the fact that the youngest part of a regenerating nerve is hundreds or thousands of times more sensitive to mechanical stimulation than is the undegenerated part (51) The threshold for electrical stimulation varies in inverse fashion probably reflecting channeling of current by the developing myelin

Possible effects of stretch might be expected to give considerable insight into structural parameters of excitability in nerve Using squid axons it has been found that stretch up to the breaking point has little effect on such quantities as the magnitude of the external action potential the conduction velocity or the spike rise rate (4) An abrupt and reversible increase in threshold to a short shock which occurs at a certain degree of stretch of myelinated fibers may be due to sudden expansion and thinning of a surface layer with a rise in capacity but in general, single myelinated fibers show little functional change when stretched nor has it been possible to excite myelinated fibers by stretching (31 80) In this general context it may be recalled that stretch of muscle fibers has so far failed to produce any lasting change in resting potential (54)

The failure of stretch to produce any drastic changes in excitability may be due as Boyarsky also concludes to the fact that at the molecular level the excitable structure may be a spotty one occupying only certain regions of the axon surface or it may be that as the surface structure is stretched new elements enter it so as to fill any vacated spots much as certain collapsed films on a water surface will reconstitute themselves when they are re expanded

A number of other physical properties of nerve change with excitation Observations of Hill and Keynes (37) of Hill (35 36) and of Bryant and Tobias (7) showing changes in light scattering and diameter and demonstrating the directional and quantitative dependence of these changes on osmotic pressure and long axis tension have been reviewed earlier (89)

In addition Lyudkovskaya (63) has made observations on light transmission through nerve in the region of polarizing electrodes A discrepancy between these findings and those of Tobias and Solomon (93) and of Tobias (87) has not yet been resolved

Kornakova *et al* (52, 53) have shown a change in rigidity of frog nerve with activity which is interpreted as an increase in viscosity It may be recalled that Flaig (22) also concluded that squid axoplasm viscosity is increased by activity Kayushin and Lyudkovskaya (47 48) have succeeded by means of an interferometric technique in demonstrating an increase in diameter (about 0 03 per cent) of whole frog nerve with activity The change is rapid in onset with each impulse lasts some 100–150 milliseconds reaches a plateau and then falls off more slowly toward the resting level It is reminiscent of the light scattering change which has been shown to take place with single impulses in whole *Carcinus* nerve (88 89) Bryant and Tobias (7) have demonstrated with *Carcinus* nerve a small reversible shortening (*ca* 0 03 μ/mm/1 000 impulses) with activity This shortening is enormously smaller than would be expected if it were produced by swelling to the extent found by Kayushin and Lyudkovskaya if such swelling took place in a constant volume cylinder

While these physical changes are interesting there are as yet no data naming any participating molecules Shaw and Tobias (81) have shown that some of Hill s early assumptions concerning quantitation of the light scattering change were probably in error The error was such as to make more reasonable his suggestion than an ion driven water movement is at least partly responsible for some of the effects However the mechanisms involved in these scattering changes have not yet really been clarified Poisoning with azide which prevents sodium extrusion and holds certain metabolic activities in abeyance without preventing impulse conduction does not interfere with the genesis of the light scattering response with activity but prevents recovery from it (8) Therefore the light scattering change even though it normally

long outlasts the electrical response, seems to be initiated by activity processes rather than by long lasting recovery events Finally observations by Bryant and Tobias (8) on the relation between nerve shortening and light scattering changes make it highly unlikely that these changes depend exclusively on intracellular extracellular water movements It is suggested that the increased scattering type of response with activity would be the more interesting one to study intensively

EFFECTS OF APPLYING ENZYMES TO AXONS

Both so called unmyelinated (lobster) and myelinated (frog) axons have been isolated and while still functional, have been exposed to various enzymes applied to the outside In the case of the lobster axon the enzyme solution was applied uniformly over the entire exposed outer surface in the case of the frog axon it was applied to the nodal region The axons at the same time were monitored for changes in such functional parameters as threshold conduction velocity nodal action current trans surface potentials (lobster axon only) and survival Finally the axons were fixed with OsO_4 imbedded in methacrylate and examined electron microscopically (66 90 91)

Let us consider first, the experiments with lobster axons When exposed for times up to 3 hours to sea water containing either chymotrypsin alone or trypsin plus chymotrypsin the axons become mechanically fragile sticky, and soft The latter is attested to by the fact that a microelectrode penetrates without dimpling or moving the axon Functionally however by the criteria listed above these axons are intact and to the eye (*ca* 36×) they look entirely normal When examined electron microscopically they must of course be compared with controls soaked for an equivalent length of time in the medium without the enzymes Such controls are already much changed from unmanipulated axons immediately fixed and never exposed to any artificial medium and therefore very detailed changes cannot be relied upon However an over all gross effect of the enzymes is readily discernible Thus the protease treated axon shows practically no evidence of the residual normal structure The surface lamellae are generally all destroyed and the axon appears as a ring of fragmented debris An explanation reconciling this appearance with the apparently normal functional status will be attempted after describing certain other experiments

When lobster axons are exposed to phospholipase A, they become excitable in lengths of time roughly related to the phospholipase A concentration These axons look normal to the eye (36×) and give no evidence of mechanical changes such as stickness fragility or easy penetrability Nor are any convincing structural changes seen electron microscopically This enzyme removes the alpha fatty acid from certain phospholipids

When lobster axons are exposed to phospholipase C they fail just as if they were treated with pholpholipase A Now however a characteristic change can be seen by the eye (36×) the axons look frosted as if their surfaces contain many small light scattering particles Electron microscopically the surface lamellae are more or less intact i e recognizable but they are greatly stretched and tortuous, randomly distributed among them are densely osmiophilic black particles This enzyme splits phosphoryl choline from lecithin leaving a diglyceride residue

These observations are discussed in more detail elsewhere (91) To explain them it has been assumed that there is at the axonal surface a structure

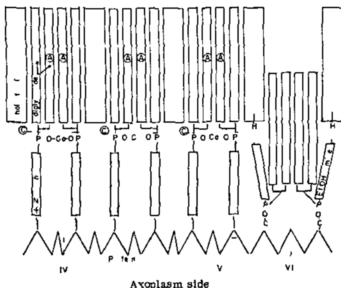


Fig 1

composed of a layer of axially oriented protein helixes coupled to a layer of radially oriented phospholipid and cholesterol molecules (Fig 1) Considering certain chemical data for the lobster axon it is tentatively assumed that the phospholipid layer will be bound to the protein by choline N^+ coupled to negatively charged protein sites by phospholipid O^- coupled to positively charged protein sites (not shown) and by Ca^{++} forming a bridge between phospholipid O^- and negatively charged protein sites (not shown) One cholesterol molecule is held between adjacent pairs of phospholipid molecules mainly by surface forces but in part by hydrogen bonding either to the amino group in cephalin or directly to regions of polypeptide bonds in the protein moiety (not shown) It is also suggested that Ca^{++} will bind pairs of neighboring phospholipid molecules to one another by complexing between their phosphate oxygens In

terms of this model the experimental findings can be rather easily rationalized as follows

Presumably proteases somehow penetrate and cleave the oriented protein helices at various sites (as IV V VI) The polypeptide fragments however are held in place by their ties to the adjacent phospholipid cholesterol film and function is maintained though mechanical strength is decreased When assaulted by OsO_4 *water alcohol and imbedding solutions, the weakened* axon breaks up into fragments and finally appears simply as a ring of granular debris

When treated with phospholipase A one of the hydrocarbon chains is split from lecithin—the dominant phospholipid in lobster nerve—and this inactivates the axon electrically, though the steps by which this end result is achieved are not known Removal of one fatty acid, is however not enough seriously to alter the structural organization of the surface layers (the split off fatty acid may even remain in the surface matrix) and no characteristic electron microscopically recognizable change is seen

Phospholipase C by disrupting lecithin also inactivates the axon but now the molecular cleavage breaks bonds which play a major role in coupling the phospholipid cholesterol layers to the protein underlay As a result the diglyceride residues and cholesterol break away from the protein and coalesce into droplets which appear as light scattering particles to the eye and as densely osmiophilic particles in the electron beam The distorted stretched remains of the lamellae retain a certain axial integrity because the protein backbones have not been split up but they lose the spatial orientation formerly impressed upon them by their lipid companion layer and now appear stretched and tortuous

It is concluded that phospholipid integrity is obligatory for normal operation of the nerve machine whereas a certain amount of destruction of surface layer protein can be tolerated The original paper (91) draws certain other tentative conclusions and also includes a hypothesis attempting to show how the trigger ing of excitation might be achieved in terms of the model (see below)

In the case of frog axons the electron microscopic correlations have not yet been extensively made The enzymes however have been tested As in the case of the lobster axon either phospholipase A or C can rapidly produce inexcitability The proteases have a minimal effect on survival if any and this is very much delayed in comparison with the phospholipase effects

OTHER PERTINENT EXPERIMENTS

It has been suggested that negatively charged sites on intracellularly fixed polyionic compounds play an important role in the selective accumulation of K over Na (55 56) Most emphasis has been placed on proteins though

recently it has been pointed out that intra axonal mucopolysaccharide might act much as an ion exchange resin (1) Sodium and potassium as well as other cations are considered to compete for the intracellular negative sites that these large molecules present and the intracellular concentration of any small ion is therefore thought to be a measure of or to be due to the relative binding energy This will in turn be a function primarily of the closeness with which the ion can approach the charged site Since hydrated K is smaller than hydrated Na it will more frequently be associated with the negatively charged sites and the K/K_0 ratio will therefore be higher than the Na/Na_0 ratio A theoretical treatment of this possibility (57) is said to utilize only intramolecular forces and not to require knowing the radii of the hydrated ions The appropriate orientation of the intracellular fixed charges is held to be important for the function of the system and this is thought to be influenced by high energy phosphate compounds such as creatine phosphate and adenosinetriphosphate The fact that ion segregation is maintained for many hours in cooled muscles whose metabolism has been poisoned with certain inhibitors would probably be thought to indicate that the ion segregation and fluxes are equilibrium processes That is energy put into the system in the form of high energy phosphate compounds could serve to orient the polyionic compounds in such a way that differential binding of ions occurs The data obtained with squid axons are not in agreement with those for frog muscle (45) Greater stores of high energy phosphate in muscle may account in part for the difference but this is probably not the whole explanation The axon may possess a different sort of intracellular anion matrix which may require a higher rate of metabolic reconstitution The association of small cations with fixed anionic sites might seem in conflict with the high mobility of cellular K (44) and with the apparent ability of cellular K to contribute fully to osmotic and vapor pressures (38) However such electrostatic associations as are postulated would not necessarily be rigid They would represent a most frequent or most probable association and the extent to which such a loose association would interfere with the movement of K from charged site to charged site as opposed to the movement from cell to external medium is not entirely clear Other pertinent data and a different point of view are presented by Simon *et al* (82) A general critique unfavorable to this point of view is that of Conway (12)

The ion exchanges of activity could be triggered or permitted by changing the form or structure of such a matrix so that fixed negative sites no longer favored K^+ over Na^+ Thus by enlarging the negative site the size difference between hydrated Na^+ and hydrated K^+ could become unimportant It is difficult however to see how current in an electrolyte solution whether applied by stimulating electrodes or by catelectrotonic spread ahead of an impulse could alter such macromolecular configurations except at interfaces

between bulk phases Indeed it is known that intracellular current alone does not excite and that only positive current flowing outward across the cell surface is effective

It has by contrast been suggested (91) that an integral part of triggering of excitation consists in driving K^+ ions from the aqueous protein solution of the axoplasm into the interface between this solution and the radially oriented surface lipids Here according to the hypothesis are found the sensitive electro static bonds between phospholipid protein and calcium which would tend to be ruptured by the intruding K^+ in such a way as to favor tangential spread ing of both the lipid and the protein moieties (Fig 1) This would, in turn affect the geometry of the subjacent axoplasm as if by a template In addition lateral spread of such surface structure would increase the total volume of trans surface aqueous pathways In other words, the surface structure has sensitive electrostatic bonds in just the right place for them to be altered by a stimulating current whether artificially or normally applied and this would result in a change in both surface structure and subjacent axoplasm structure

The finding of Geiger *et al* (27) that non protein nitrogen rises and that lipid and nucleic acid nitrogen decrease in stimulated brain suggests that changes in large molecules do occur That some structural change of protein takes place during excitation is also indicated by work of Ungar *et al* (94) which suggests that a certain amount of neural protein is reversibly denatured during activity The time course of reversal is similar to that for recovery from the optical changes accompanying activity (7 8) It would be interesting to see whether the reversal of denaturation noted by Ungar would be blocked by dinitrophenol or azide as are the increase in oxygen consumption with activity (6) the recovery from the light scattering changes (8) and the extrusion of Na and reaccumulation of K which normally follow activity If so the finding of such an attribute common to certain structural ionic and metabolic parameters of activity might suggest new insights into their meaning

Interpretation of trans surface water movements could also be considered in this context However, most of the data on water transport have been interpreted in terms of a porous membrane at the cell surface Comparison of rates of water entrance into cells under the influence of osmotic pressure gradients and as a result of a simple diffusion gradient gives some indication of what may be termed "equivalent pore size" (50 72) Values of equivalent pore radius ranging from 3 A in red blood cells to 16 A in squid giant axons have been suggested (67 83) When cells are placed in a medium labeled with deuterium tritium or O^{18} equilibration with the medium is very rapid with a half time of a minute or less for whole frog nerve (92) and a matter of milliseconds for erythrocytes (83) The existence of pathways in which

water moves extremely freely would therefore seem likely Mullins has carried the analysis of pore size and configuration further (65) The varying activity of certain compounds such as some narcotics and DDT and their homologues is interpreted in terms of the ability of models of these molecules to fit into pores of various sizes The pores are considered to be the interstices between large spherical membrane molecules which are packed to within about 2 A of one another When these membrane molecules have reactive sites on them such as the esteratic and anionic sites of choline esterase further specificity is conferred on the model Mullins (65) points out that many of the data he presents especially on narcosis can be interpreted in terms of either a homogeneous lipid film or a porous membrane at the surface He feels however that certain of the evidence favors the latter

Teorell (86) has obtained interesting periodic variation in water movement in a model system When a constant current is passed through a cell composed of solutions of unequal NaCl concentrations, separated by a porous glass membrane, damped and even undamped oscillatory movements of water and NaCl occur Changes in pressure in the two sides of the cell and changes in membrane voltage and resistance accompany the water movements Teorell suggests that for the understanding of transport and electrokinetic phenomena the pressure variations in biological systems are an important and as yet little analyzed parameter

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CHAPTER 20

Studies on Nucleolar Function*

Hewson Swift

NEW insights into the chemistry of biosynthesis force us to reinvestigat old problems of cell function One such problem concerns the role of th nucleolus in protein synthesis It has been known for many years that nucleo are enlarged in growing and secreting cells Regenerating tissues—for exampl of moss (38) and mammalian liver (86)—have markedly larger nucleoli tha their normal counterparts An enlarged nucleolus has been considered a majo criterion of a rapidly growing tumor (60) There are numerous observations i the literature connecting nucleolar enlargement with protein synthesis and ye concerning the actual causal relationships the literature provides us with man theories and few facts

This chapter discusses the nature and behavior of nucleoli in growing tissue First current ideas on the origin of nucleoli in the mitotic cycle are re examined and second, some changes in nucleolar morphology and composition are de scribed associated with changing conditions of cell growth The materia presented constitutes a few selected examples that bear on the problem o nucleolar function Much of the data will appear elsewhere in more extensiv form

MATERIALS AND METHODS

Observations on normal onion tissues were made on roots grown in ta water For irradiation studies with slow neutrons onion sets (var Ebenezer were treated for 16 minutes in the heavy water pile of Argonne Nationa

* This paper includes data from several studies undertaken in our laboratory which will be pub lished elsewhere in more extensive form The author wishes to thank a number of associates an graduate students for permission to include unpublished data in some cases from work in progres Regenerating rat liver studies were made by Dr Ellen Rasch and Dr Ruth Kleinfeld Determinatio on nucleoproteins in bean nucleoli were made by Dr John Woodard who also prepared sections o bud primordia HeLa cell studies were made by Dr Stephen Fantelik Studies of *Drosophila* salivar glands were made by Dr Miriam Schurin Mrs Agnes Stroud prepared the colchicine treated ra tissues and Mr Wayne Ferris assisted with the electron microscopy Particular thanks are due t Dr Rasch for invaluable assistance in the preparation of this manuscript This work was supporte by USPHS Grant C 1612 and the Wbett Memorial Fund

Laboratory and then soil planted 4 days later Roots were collected 96 hours after planting (76) Onion tissues were fixed in acetic alcohol for 1 hour in Navashin s fluid or in 1 per cent chromic acid at pH 3 0 for 24 hours Third instar larvae of *Drosophila virilis* were homozygous for translocation pe^{m} t (4) Salivary glands were smeared in 45 per cent acetic acid immediately frozen on dry ice for cover slip removal and then transferred to 70 per cent alcohol Larvae of *Chironomus* sp were collected from ponds in the Chicago region tissues were fixed for 1 hour in acetic alcohol HeLa cells were obtained in flask culture from Microbiological Associates and were starved in Hank s salt solution or grown in standard Eagle s medium Cover slips with cells attached, were fixed 5 minutes in acetic alcohol (68) Liver regeneration studies were carried out on 5 month old male D 52 strain rats the hepatectomy performed as described by Higgins and Anderson (41) Karyomeres were produced with colchicine injections (0 15 mg/100 gm body weight) given 24 hours after hepatectomy animals were sacrified 24 hours later Rat tissues were acetic alcohol fixed for 1 hour For all electron micrographs tissues were fixed for 1 hour in 1 per cent osmic acid adjusted with veronal acetate or McIlvain buffer to pH 7 4 and imbedded in methacrylate sectioned on a Porter Blum microtome and examined with an RCA EMU 2d microscope

Desoxyribonuclease (crystalline Worthington) treatment was carried out on acetic alcohol fixed tissues in an 0 001 per cent solution with 0 003 *M* $MgSO_4$ added adjusted to pH 6 0 with 0 1 N NaOH for 1 or 2 hours at room temperature Appropriate controls were used (89) Ribonuclease (crystalline Armour), 0 01 per cent was used at pH 6 5 for 1 hour at room temperature Tissues were stained for RNA and DNA with azure B 0 025 per cent in McIlvain s buffer at pH 4 0 (33 89) Photometric determinations of RNA–azure B binding were made in visible light as described by Swift and Rasch (91) Acid dye staining of nucleoli was performed on tissues fixed for 24 hours in Navashin s fluid or in 1 per cent chromic acid at pH 3 0 Sections were stained for 1 hour in 0 5 per cent fast green at pH 2 0 and were differentiated for 30 minutes in phosphate buffer at pH 6 0 Sudan black B staining was used on chromic acid–fixed tissues and followed the method of Chiffelle and Putt (23) Total proteins were stained with the Millon reaction (72 77) or with fast green following acetic alcohol fixation

OBSERVATIONS

I Nucleolus Chromosome Relationships

1 *The mitotic cycle in onion roots* —In telephase nuclei of onion it is possible to distinguish three types of RNA containing material For purposes of convenience these may be called (1) spindle remnant (2) nucleoli and (3) chromosomal (but extra nucleolar) ribonucleoprotein In onion roots as in

many cells the spindle substance contains RNA as evidenced by its strong characteristic ultraviolet absorption and basophilia both removable by ribonuclease This RNA containing material lies between the chromosomes (Pl I Fig 4) It shows no obvious tendency, however, to be concentrated around them as a specific coat or matrix, except in tissues that appear poorly fixed In electron micrographs of tissues fixed in buffered osmic acid no matrix material is apparent (Pl VIII, Fig 35) After treatment with desoxyribonuclease the telophase chromosomes are unstained and are sharply outlined by this substance as seen in Plate I Figure 3 When the nuclear membrane forms, some spindle remnant remains inside the nucleus This material persists into early interphase as an irregular array of wisps and particles that grow fainter and eventually disappear (Pl I Figs 2, 3, 4) We have seen almost no tendency for these areas to coalesce In most early interphase nuclei of onion root they are still visible when the nucleoli have grown to their full early interphase volume Under the electron microscope the areas of spindle remnant in telophase contain numerous fine particulates in a loose arrangement closely resembling the cytoplasm (Pl VII Fig 33 Pl VIII Fig 36) Although this material may well be reused after breakdown for other processes this fraction has no obvious morphological connection with chromosomes or nucleoli It seems probable that its occurrence is largely adventitious in telophase and early interphase nuclei

What we have called spindle remnant may readily be confused with nucleolar material in telophase since both fractions stain alike with basic dyes They may be distinguished however with other stains When onion root tips are fixed for long periods (e g 24 hours) in chromic acid formalin fixatives the nucleoli stain both with Sudan black B and fast green whereas the spindle substance does not (Pl II Figs 5 6) It is difficult to interpret in biochemical terms the effects of such fixation and staining It seems likely however that the fixative retains lipids or phospholipids in the nucleoli forming an insoluble chromate lipid complex that withstands dehydration and imbedding procedures (3) Sudan black staining, under properly controlled conditions has been considered an indication of lipids The fast green staining of nucleoli is strongly pH dependent and probably is influenced by the positive charge of the chromate lipid complex and probably also by the free amino groups in the highly concentrated nucleolar protein Nucleoli have been reported to show positive lipid reactions by several authors particularly Albuquerque and Serra (1) Specific acid dye staining of nucleoli has also been reported by Semmens and Bhadhuri (82 83) Their staining procedure in our hands is also dependent on chromate fixatives and able to distinguish nucleolar material from spindle substance It seems likely that these tests are indicating an as yet uncharacterized lipid fraction present in nucleoli, possibly protein

bound which does not occur in the remnant spindle material Without chromation of tissues and proper differentiation, fast green, being a general protein stain also stains spindle material

In roots stained with fast green, the forming nucleoli are occasionally first visible in telophase nuclei each as two fine granules in intimate association with the nucleolus forming chromosome as described by Heitz (39) A similar pair of granules was seen by Dearing (27) in amphibian epidermal nuclei

In electron micrographs of late anaphase and telophase nuclei small dense areas are visible in intimate association with the chromosomes In their structure and density these areas somewhat resemble the nucleolus both contain a few characteristic highly electron dense particles of variable size As shown in Figures 32–36 (Pls VII and VIII) these areas are more dense than the chromosomes, while the material we have identified as spindle remnant is less dense With azure B after desoxyribonuclease treatment these regions are visible with the light microscope as fine particles lining the chromosome Occasionally they appear to be paired as in the forming nucleolar rudiment This component which we have called 'chromosomal ribonucleoprotein or RNA is difficult to distinguish from the degenerating spindle substance but at least in some cells is more heavily basophilic and seems attached to the chromosomes and not merely lying in the interchromosomal spaces It seems unlikely that chromosomal RNA merely represents spindle substance that has been passively concentrated between the chromosomes in telophase Chromosomal RNA appears in anaphase before the chromosomes become contiguous and it possesses its characteristic fine structure from its first appearance The particles are not visible in our fast green or Sudan black preparations possibly because they are too small to show a positive reaction They may be similar in part to the 'pronucleolar substance described by Rattenbury and Serra (79) and also similar to the granular component described by Estable and Sotelo (30) as lying along the nucleolonema in telophase nuclei stained by silver impregnation In some cases the fine granules form rows along the teleophase chromosomes although in our preparations stained for RNA we have found no connecting filament as described by these workers In the onion these fine granules are visible only during early interphase In some preparations of the related genus *Nothoscordum* however numerous very fine RNA containing particles may persist throughout interphase surrounding the areas of DNA containing material In this respect this fraction resembles the chromosomal RNA we have described for *Tradescantia* pollen and amphibian tissues (92)

Throughout the rest of interphase in onion root nuclei the only stainable RNA occurs in the nucleolus After desoxyribonuclease treatment and azure B staining areas occupied by chromatin are completely unstained (Pl I Figs 3 4) Where desoxyribonuclease treatment is prolonged however or the

enzyme contaminated with bacteria, chromatin may become basophilic with ribonuclease extractable material. We have interpreted this staining as a diffusion artifact (88). The absence of stain in interphase nuclei after desoxyribonuclease may indicate the absence of measurable chromosomal RNA. It is also possible, however, that RNA may be present in a form in which the phosphoric acid groups are masked by proteins. The increased acid dye binding after ribonuclease reported by Kaufmann *et al.* (49) and ultraviolet absorption studies on lily nuclei (87) suggest the latter possibility. Ultraviolet absorption studies need to be made on these tissues to test this possibility.

Throughout interphase, onion nucleoli show little change in morphology. Nucleolar number is higher in early interphase than in late interphase because of the tendency for nucleoli to fuse (text Fig. 1). Where mitosis is blocked

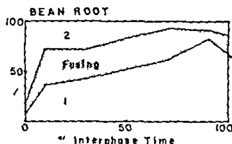


FIG. 1.—Nucleolar number during interphase in bean root (*Vicia faba*). Interphase time was estimated by nuclear volume. Telophase values are included at 0 time and prophase values at 100. Nuclei were scored as containing one or two nucleoli or with one nucleolus which was bipartite, apparently in the process of fusing. The slight increase in nuclei with "fusing" or two nucleoli in prophase may indicate that fused nucleoli tend to be pulled apart as the chromosomes shorten. From Woodard (unpubl.).

by radiation, thus prolonging the interphase time, the nucleolar number in the normal unfragmented nuclei becomes still lower (90). Nucleoli contain small vacuoles of non-staining material. Occasionally these are connected with the nucleoplasm through a small opening. In some preparations nucleoli are surrounded by a large vesicle which forms a space between the chromatin and the nucleolar margin. It is largest in root tips fixed in 1 per cent chromic acid, smaller with acetic alcohol fixation, and usually absent in cells fixed in buffered osmic acid, although it has been reported by Chayen (21) from living cells.

In prophase the onion nucleolus changes from a roughly spherical to an irregular shape with pointed projections ending on chromosomes and in some cases approaching the nuclear membrane as well (Pl. I, Fig. 3). In interphase and early prophase portions of many chromosomes appear in contact with the nucleolus. It is possible that, when chromosomes shorten and straighten

during prophase, these points of contact are pulled away from the main body of the nucleolus forming the pointed projections Smaller projections are visible on the nucleolar margin of most cells throughout interphase as indicated in electron micrographs (Pl VIII Fig 37)

In late prophase slightly before the time that basophilic spindle material is forming outside the nuclear membrane the chromosomes become outlined with RNA containing material (Pl I Figs 1 3) It has been suggested that this chromosomal RNA fraction forms from the degenerating nucleolus (37) In onion this seems unlikely for several reasons The nucleolus shows great variation in its rate of dissolution Occasionally it persists until metaphase with almost no diminution in volume Chromosomal RNA in late prophase is a much more constant fraction Also it shows no gradient away from the nucleolus as might be expected if it was of nucleolar origin It is possible that this material may represent cytoplasmic RNA entering the nucleus through the rupturing membrane but this seems unlikely since the nuclear membrane normally appears intact when this fraction is first visible In several cell types RNA containing material has been described as lost from the chromosomes during mitosis (45 81) This fraction in onion may represent a similar phenomenon or may be material newly synthesized as in the prophase of grasshopper spermatocytes (92)

2 *Extra nucleoli*—In most cells nucleoli first appear in late anaphase or telophase at specific sites on specific chromosomes The relationship between nucleoli and certain chromosomes was described in grasshopper tissues by Carothers (19) and Wenrich (97) and in hyacinth by de Mol (28) However the importance of specific chromosome loci in the formation of nucleoli in telophase was first clearly established by Heitz (39) and McClintock (61)

During the formation of maize pollen McClintock described a pool of aceto carmine stainable material around the chromosomes at telophase I interkinesis and telophase II She suggested that this matrix material is organized into nucleoli by the specific chromosome regions This concept was supported by a study of abnormal maize microspores containing only a portion of the normal organizer region because of a translocation In these cells the nucleolar substance was diffuse in many separate bodies rather than in the usual single nucleolus

It is important to our understanding of nucleolar function to determine whether the nucleolus forms from material sloughed off the chromosomes at telophase or whether it is a product of a specific chromosome locus In one case we would expect the nucleolus to contain the non specific products of mitosis but in the other to represent a specific gene product possibly with a specific role in cellular synthesis

To investigate the problem of nucleoli and chromosomal matrix two cases

have been studied in which nuclei were experimentally fragmented into a number of separate nuclear vesicles or karyomeres, each surrounded by a nuclear membrane If nucleoli arise from matrix material, as a product of chromosomes in general we would expect each karyomere to contain a nucleolus If on the other hand nucleoli are synthetic products of specific chromosome loci then some karyomeres might be expected to lack such loci and therefore also to lack nucleoli In one case onion bulbs were treated with slow neutrons At doses which produce chromosomal fragmentation but no or infrequent cell death viable interphase cells containing several karyomeres occur in large numbers (76) These karyomeres arise in telophase from acentric chromosome fragments, and in some cases can be seen to proceed into subsequent prophase more or less in phase with the parent nucleus A number of the interphase karyomeres (about 15 per cent) contained no nucleoli whatever, strongly suggesting that the ability to form nucleoli is possessed only by certain regions of the chromosome set In most cases however the fragmented nuclei contained more nucleoli per cell than did the controls (Pl II Figs 7–9)

Somewhat similar results were obtained in regenerating rat liver nuclei which were made karyomeric through colchicine disruption of mitotic spindles (16) Cells blocked in metaphase eventually proceed on into interphase but in the absence of the spindle the chromosomes disperse and form anywhere from two to about twenty karyomeres (Pl III, Figs 12 13) As in onion the majority of these form their own nucleoli and average many more nucleoli per cell than do normal nuclei A few karyomeres however were without nucleoli as shown in Plate III Figure 14 Presumably these are formed from one or more chromosomes not containing a nucleolus forming region The situation is less clear than in irradiated onion cells since the karyomeres are often small (less than 2 μ in diameter) and there is a slight amount of necrosis in the tissues Figure 14 in Plate III shows a group of karyomeres however in a cell that appears to be normal except for the fragmented nucleus

In karyomeric cells of both onion and rat liver we can conclude that every chromatin fragment does not necessarily form a nucleolus in telophase and therefore that the nucleolus does not form from matrix material collected from all the chromosomes Since karyomeric cells contain more nucleoli than their normal counterparts however we must also conclude that the chromosome set contains a number of potential nucleolus forming loci, active in isolated karyomeres but not active in nucleolar synthesis in most normal cells

Under certain conditions however extra nucleoli or nucleolus like structures can arise in normal nuclei The simultaneous formation of many nucleoli was described in oocytes of several species by Jorgensen (46), in snail oocytes by Fahmy (31), and in rat pronuclei by Austin (2) An extra pair of small nucleoli forms in spermatocytes of the gerbil, an African rodent (94) and numerous

nucleolus like bodies arise on the chromosomes of early grasshopper oocytes (92) In *Chironomus* cold treatment causes small nucleolus like bodies to appear at new chromosomal loci of the salivary chromosomes normally occupied by puffs (8) A similar situation in plants is shown in Plate III Figures 10 and 11 In the lateral bud primordia in *Tradescantia* growth is blocked by auxins from the growing shoot apex The arrested lateral buds contain enlarged nuclei which from their DNA content are known to be diploid (66) The surrounding leaf tissues all contain the usual one to six nucleoli but up to fifteen small nucleoli have been counted in the large nuclei of the bud primordia

In these cases where extra nucleoli arise the nuclei are large or in the process of enlarging as compared with other tissues They are not necessarily rapidly growing or synthesizing tissues In fact in the lateral bud primordia of *Tradescantia* cell growth is completely inhibited

3 *Nucleoli and chromosomal RNA* —In nuclei of certain cells basic dye binding after desoxyribonuclease reveals the presence of a great deal of RNA in addition to the nucleoli The liver nuclei of salamander larvae for example contained about four times as much RNA associated with chromatin as with nucleoli when estimated by photometry with azure B (92) In the lamp brush chromosomes of *Triturus* oocytes RNA was found by Gall (35) in the lateral loop projections These were shown to vary in their morphology some containing extremely fine particles barely visible under the phase microscope while others contained spherical granules the size of small nucleoli (34) It thus seems likely that chromosomal RNA in amphibian oocytes is associated with particulates varying in size and at least in some regions resembling the nucleolus in certain morphological characteristics

We were interested in investigating the morphology and distribution of chromosomal RNA in the giant polytene chromosomes of *Drosophila* and *Chironomus* salivary glands Here somewhat as in amphibian lamp brush chromosomes RNA was found associated with a variety of structures in some cases closely resembling the nucleolus Mid and late third instar larvae of *Drosophila virilis* were smeared in 45 per cent acetic acid and then stained with azure B In most preparations treatment with desoxyribonuclease caused the non specific adsorption of RNA Consequently slides were usually studied without desoxyribonuclease treatment and the RNA was localized by its characteristic metachromasy (33) RNA was found to be associated with a small number of chromosomal loci varying from barely detectable amounts in some bands to grossly enlarged puffs (Pl IV Figs 15 20) Most RNA containing bands occurred at thickened regions of the chromosome Often the RNA band occurred contiguous to DNA so that a single band stained for DNA on one edge and for RNA on another (Pl IV Figs 17–18) In some puff regions RNA containing material appeared to extend from the chromo

some out into the interchromosomal material It is possible that in the process of smearing some substance could be displaced from the chromosomes but similar material was also seen in sections The *Drosophila virilis* strain used contained a small nebennucleolus ' In some cases these were connected to RNA containing bands (Pl IV, Fig 23) In a few cells the largest puff was greatly extended from the chromosomes becoming spherical in outline In this form it closely resembled the nucleolus in both RNA concentration and morphology

The nucleolus forming locus in *D virilis* is located within the chromocenter We found no evidence for RNA within the heterochromatin but frequently at the point of nucleolar attachment DNA containing material extended from the chromocenter into the nucleolus as described for *D melanogaster* by Heitz (40) and Kaufmann (47, 48)

The relation between nucleolus and chromosome is more readily studied in *Chironomus* salivary glands As described by several authors (6 50 74) in certain species the shortest chromosome—chromosome IV—contains the main nucleolus an immediately adjacent puff region (the Balbiani ring) and also a variable terminal mass of nucleolus like material In the nucleolus forming region as described by Bauer (6) the chromosome strands may be somewhat expanded and displaced by the nucleolar substance and some DNA containing material frays out into the nucleolus A greater expansion of the chromosomal strands occurs in the Balbiani ring (8) and also in the terminal mass In our preparations, chromosome IV stained intensely for RNA in the terminal puff and Balbiani ring as also reported by Beermann (9) RNA was also localized in a band across the nucleolus forming region which spread out into the nucleolus (Pl IV, Figs 21 22) This band appeared slightly different in texture from the nucleolar substance itself Several small nebennucleoli also appeared to be associated with RNA containing regions on chromosomes III and IV (Pl IV Fig 21) More studies like those of Beermann (8) are needed on the variation of these structures with changing cell states

II *Nucleolar Variation in Interphase*

1 *Changes in shape and number* —Nucleoli frequently show changes in shape during different stages of cell growth as shown for example by Lesher (53) in different growth stages of *Drosophila* salivary nuclei Many marked shape changes have been described in nucleoli of invertebrate oocytes including fragmentation and the spinning-out of nucleolar material into bands or filaments (e g 46) In young grasshopper oocytes for example the nucleolus appears typically spherical but later expands into a loose granule lined filament (92) Less bizarre but nevertheless consistent, shape changes occur

in many tissues during cell growth These seem important in understanding the relation between nucleoli and their surrounding structures

Cell cultures of human cervical carcinoma (strain HeLa) when rapidly growing in Eagle s complete medium characteristically showed the nucleolar shape indicated in Plate V Figure 24 Although these cultures normally displayed considerable variation in most cells the nucleolar substance was distributed in one or more very irregular central masses Most nucleoli contained characteristic pointed projections which extended out into the nucleus or joined with other nucleoli When cells were starved for 72 hours on Hank s salt solution lacking necessary amino acids the nucleolar substance was distributed in from 1 to 8 small spherical bodies per cell (an average of 3 7) as shown in Plate V Figure 25 Cultures were then returned to supplemented medium and the cells were sampled periodically by removing cover slips placed on the bottom of the culture vessel After 6 hours in new medium the nucleolar number dropped to an average of 1 7 The nucleoli were markedly enlarged but still essentially circular in outline (Pl V Fig 26) Their original morphology with radiating projections returned after 24 hours on supplemented medium as shown in Plate V Figure 27

Somewhat similar changes in shape have been observed in nucleoli of rat liver during regeneration following partial hepatectomy Nucleoli in young adult animals are normally approximately spherical Fifteen hours after hepatectomy when nucleolar RNA was rapidly increasing liver nucleoli became irregular in outline as shown in Plate VI Figure 29 This was partly because of nucleolar fusion but also nucleolar margins showed numerous small radiating projections Many nucleoli at this time had a peripheral position against the nuclear membrane At 24 hours the nucleoli were maximally enlarged but most were again spherical in outline (Pl VI Fig 30) At 36 hours the nucleoli again became less prominent and more numerous The cells were somewhat distorted by vacuolation at this time (Pl VI Fig 31)

Marked changes in nucleolar number were also apparent during regeneration Nucleolar number was determined only in tetraploid nuclei as selected by their relative diameters when compared with diploid and octaploid nuclei (78) In control tissues these nuclei contained a maximum of 4 nucleoli and an average of 2 8 As shown in text Figure 2 the nucleolar number in tetraploid nuclei fell to an average of 1 2 at 24 hours and returned to the control value after 48 hours Reduction in nucleolar number was apparently brought about by two processes —fusion and disappearance When nucleoli appeared dumb bell or hour glass shaped it was assumed that they were in the process of fusion Such fusion figures rose to a maximum at 20 hours (text Fig 2)

Of greater interest was the reduction in nucleolar number through the disappearance of one two or three nucleoli with the increase in size of the

fourth In some nuclei one nucleolus was large and the remaining one to three nucleoli were much smaller in some cases close to the limit of microscope visibility (Pl VI Fig 29) The number of nuclei showing one large nucleolus with one to three small nucleoli (below 1 μ in diameter) increased during regeneration from 5 to 21 per cent at 24 hours (text Fig 2) It thus seems likely that in addition to nucleolar fusion the nucleolar number was also reduced in some cells by the decrease in size and eventual disappearance of all but one of the nucleoli An alternate explanation would be that these

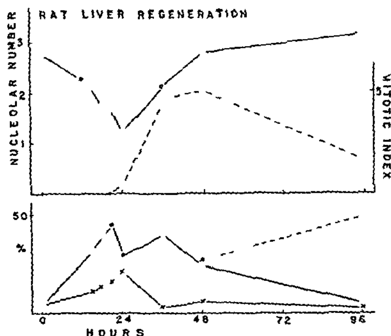


FIG 2 —Mitotic index and nucleolar behavior during rat liver regeneration The upper graph shows the average number of nucleoli per tetraploid cell (*solid lines*) and mitotic index (*dotted lines*) Lower graph shows per cent tetraploid nuclei containing four nucleoli (*dotted lines*) the per cent showing two or more nucleoli in the process of fusion (*solid lines circles*) and the per cent showing one large nucleolus and from one to three minute nucleoli below 1 μ in diameter (*solid lines crosses*) Mitotic index values from Kleinfeld (51) other data from Kasch (unpub)

minute nucleoli represent extra nucleolar bodies not normally present This seems unlikely since the number of bodies almost never exceeded a total of four Five nucleoli were seen in only 0 2 per cent of the nuclei scored

The increase in nucleolar number at from 24 to 96 hours after hepatectomy parallels the increase in mitotic index (text Fig 2) It thus seems likely that this represents the tendency for the majority of these cells to enter interphase after division with four nucleoli

In nucleoli of bean meristem fusion appeared to occur primarily as a function

of time In text Figure 1 the number of nucleoli was plotted against per cent interphase time as determined by nuclear volume Except for a sudden rise immediately following telophase the number of fused nucleoli increased in a roughly linear fashion

2 *Changes in chemical composition* —In starved and re fed HeLa cells and in regenerating rat liver, the amounts of RNA per cell were estimated by photometric determinations of the amounts of azure B bound to the RNA Proteins were similarly determined with the Millon reaction (72 77) or by

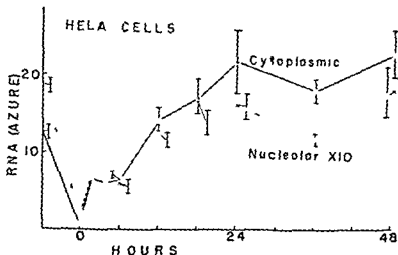


FIG 3 —Photometric determinations on nucleolar and cytoplasmic RNA in HeLa cells grown in cell culture Cells were starved for 72 hours in Hank s salt solution supplemented medium was then added at time 0 Each point represents the mean of from ten to twenty determinations on the total RNA per nucleolus or cytoplasm per cell Vertical lines show standard errors Only cells with single nucleoli were measured Cytoplasmic RNA was determined as the average of three extinction measurements taken through different cytoplasmic regions of each cell The average extinction was multiplied by the cytoplasmic area as determined from planimeter tracings of camera lucida drawings Data of Pantelik (68)

fast green binding Only cells with single nucleoli were measured to facilitate the determinations and reduce the errors associated with volume estimation For details of the techniques employed see Kleinfeld (51) Swift and Rasch (91) and Pantelik (68)

In cell cultures starved in salt solution for 72 hours about 90 per cent of the RNA was lost from nucleoli and cytoplasm (text Fig 3) but only about 50 per cent of the protein During the subsequent refeeding the RNA component of both the nucleolus and the cytoplasm was restored to normal levels more rapidly than the total protein This resulted in the fluctuating RNA protein ratios graphed in text Figure 4 showing a drop on starvation followed by a

rise to a maximum at 24 hours, and then a return to roughly normal levels after 48 hours

A somewhat similar pattern was found during rat liver regeneration The RNA per tetraploid cell rose markedly in both nucleolus and cytoplasm during the first 24 hours following the operation and then decreased in association with cell division and the resulting reduction in cell volume During the first 48 hours the RNA protein ratio in nucleoli increased In the cytoplasm however the ratio first fell to a low at 24 hours and then rose to above normal at later stages of regeneration (text Fig 5)

Several points of interest are shown by these determinations The RNA increase in these cells is essentially parallel in nucleolus and cytoplasm, but in

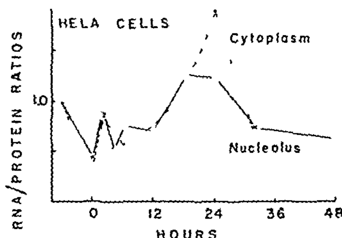


FIG 4 —Relative RNA protein ratios in HeLa cells grown in cell culture Cells were starved for 72 hours in Hank s salt solution supplemented medium was then added at time 0 RNA values used in the ratios are those graphed in Fig 3 Protein values were made from the same preparations using the Millon reaction after removal of the basic dye Each point represents the mean of (from five to twenty) *measurements Data of Pantelik (68)*

liver the nucleolus slightly leads the cytoplasm Although clearly one cannot safely generalize from these two examples one may roughly analyze these systems in terms of two processes (1) the formation of new RNA and (2) the utilization of RNA in protein synthesis In HeLa cells step 1 apparently proceeded faster than step 2 i e RNA was restored to normal levels ahead of the protein components *In liver cytoplasm however with the normal RNA* components already present step 2 seems to have proceeded slightly faster than step 1 so that the RNA protein ratio at first fell In support of this concept that protein synthesis may have occurred first at the expense of pre existing RNA is the characteristic dissolution of the basophilic clumps of liver ergastoplasm during the early stages of regeneration (Pl VI Figs 28 29)

It is apparent from these determinations that nucleolar enlargement in synthesizing cells is a complex process and does not involve merely the accretion of a single substance of fixed ribonucleoprotein composition. In fact, in addition to the marked shift in RNA protein ratios there was also a drop in the concentration of both RNA and protein during the first 24 hours of regeneration. This may be associated with the "loosening" of the structure of the nucleolus as seen under the electron microscope discussed below.

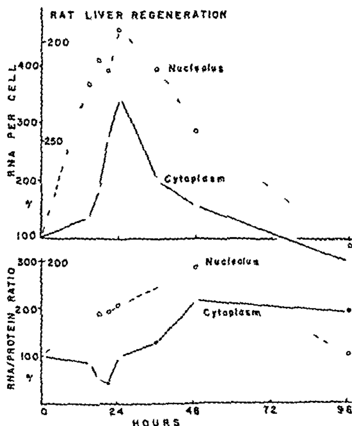


FIG 5.—RNA and protein relationships during liver regeneration. Upper graph indicates the amounts of RNA per cell in nucleolus and cytoplasm as determined with azure B. Each point represents the mean of from fifteen to forty determinations. Data are graphed as per cent change as compared with control livers removed in hepatectomy. Left ordinate applies to nucleolar values, right ordinate to cytoplasmic values. Lower graph indicates RNA protein ratios made on the same tissues. Proteins were determined by the Millon reaction (cytoplasm) or with fast green (nucleoli) and compared with the RNA values graphed above. Ratios are plotted as per cent change as compared with control livers removed at hepatectomy. (Data from Rasch and Kleinfeld unpubl., cf. Swift et al. (92) where less extensive data are given. RNA values for late stages of regeneration are lower than previously reported since cell volume was here determined by counting the number of parenchymal cells per unit of section area instead of by point hits.)

Nucleolar RNA and protein were also measured throughout interphase in bean roots (text Fig. 6) with interphase time computed by nuclear volume. These determinations showed a parallel increase in both components in interphase and no change in the RNA protein ratio. It is of interest that, in bean telophase nucleoli both RNA and protein are apparently formed at equal rates.

3. *Nucleolar fine structure*.—It is the purpose of this section merely to mention two aspects of nucleolar fine structure which have a bearing on points

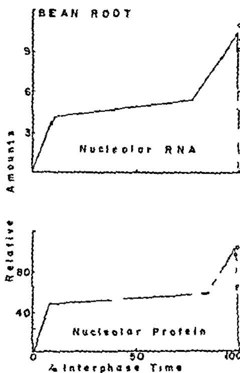


FIG. 6.—Photometric determinations on nucleoli during interphase in roots of *Vicia faba*. RNA was determined with azure B after desoxyribonuclease treatment. Total protein was determined with fast green. Interphase time was estimated by nuclear volume determination. Each point represents one measurement of the total RNA or protein in *one nucleolus*. Only cells with single nucleoli were measured. (From Woodard, unpubl.)

considered above. These are first, morphological variation in nucleoli associated with differences in RNA and protein concentration, and second, the stellate appearance of nucleoli under certain conditions possibly associated with the pointed projections seen in onion, liver and HeLa cells as described above.

Nucleoli are seldom uniform in fine structure. In most cases variations are seen in electron density and texture in different parts of a single structure. In Plate IX, Figures 39 and 40 of a *Drosophila* salivary gland nucleolus in

addition to several small vacuolar areas of low density two distinct regions are evident One with a high concentration of fine particles is largely peripheral in location The other, with more uniform texture occupies a central position There is a suggestion of fine convoluted filaments in both sections but in the outer region the filaments appear to be lined with particles also evident in the onion nucleolus shown in Plate VIII, Figure 38 From methacrylate sections of *Drosophila* nucleoli cut at 1 μ and stained for the light microscope with azure B it was evident that RNA was more concentrated in the outer portions associated with the particle containing areas

Amphibian liver and pancreas cells frequently show the nucleolar structure seen in Plate IX Figure 41, with a compact portion and an area of isolated bodies that may represent sections through an irregularly twisted thread In this nucleolus from *Ambystoma* pancreas it seems probable that the compact portion contained a larger concentration of RNA than did the more diffuse area

Figures 43–46 (Pl X) show nucleoli from normal and 24 hour regenerating rat liver cells There is wide variation in nucleolar morphology between different cells of this tissue so that these few micrographs cannot properly represent the range in form encountered At 24 hours many nucleoli appeared less compact than in the controls with more distance between the dense threadlike areas This loosening of structure possibly admitting nucleoplasm within the nucleolus may be associated with the decreased RNA and protein concentrations mentioned above Although nucleoli from regenerating tissues retained their roughly threadlike structure, they showed much more variability in electron density than did the controls as seen in Plate X Figures 45 and 46

Similar threadlike components of nucleoli were reported by Borysko and Bang (13) and Bernhard *et al* (10) and have since been found in electron micrographs of nucleoli from many tissues In a number of cases however this structure is not evident It is not visible in the onion tissues shown in Plate VIII Figure 38 or in the *Drosophila* salivary nucleoli in Plate IX Figure 40 There is a possible relation between such threadlike structures and the nucleolonema seen with the light microscope by Estable and Sotelo (30) The fine particulate component of nucleoli was described by Porter (73) By analogy with similar particulates in the cytoplasm these probably represent the site of nucleolar RNA (5 67)

The nucleolar margin is frequently irregular containing small projections as seen for example in the onion and *Drosophila* nucleoli in Plates VIII and IX Figures 38 and 40 In some cases under favorable conditions small filaments approximately 20 mμ in diameter run perpendicular to the nucleolar margin joining the nucleolus with the surrounding nucleoplasm (Pls VIII–X Figs 38 40 42 45 46) These occasionally give the nuceloli a radial or stellate appearance It is unlikely that this orientation is caused by nucleolar shrinkage

during fixation since radial areas are frequently interspersed with regions that are differently oriented The radial projections mentioned above in onion HeLa and liver nucleoli also give these nucleoli a radial orientation under the light microscope Such projections are clearly visible in living HeLa cells with the phase microscope These projections may be correlated with the radial filamentous orientation seen in electron micrographs but more careful comparisons preferably on the same cells, are needed

DISCUSSION

From the data presented here four conclusions can be made concerning nucleolar function First, we have refuted the concept that the nucleolus is 'organized from matrix material in telophase The droplets of material interpreted by most authors to be stages in the aggregation of nucleoli we consider to have no direct connection with nucleolus formation The multiple 'diffuse nucleoli forming when organizers are disrupted are best interpreted as the result of competition Second as an alternative hypothesis we have suggested that the nucleolus is a synthetic product of a chromosome locus one of a number of forms in which RNA containing materials occur in association with the chromosomes Third the 'competition between nucleolar forming sites for certain unknown components influences in part the size and number of nucleoli during cell growth Fourth changes in nucleolar form and composition associated with altered synthetic states of the cell, lead us to the conclusion that the nucleolus is a site of protein synthesis possibly of material necessary for the formation of cytoplasmic RNA

I *Nucleolar Formation*

In a consideration of telophase reconstitution of nucleoli Vincent (95) concluded An examination of the data suggests that the nucleolar materials may have a different origin during the time of telophase reconstitution than during interphase Such a dual hypothesis results from a realization that the organizer concept which considers the nucleolus to be formed from non *specific products of mitotic chromosomes is in partial contradiction to the large* body of data that relates nucleoli with the interphase growth of the cell This contradiction has led us to re examine the two basic observations on which the organizer concept was based namely, the role of matrix in telophase and the diffuse nucleoli obtained when nucleolus-forming loci were disrupted (36 61)

Nucleoli were considered by Darlington (25) 'merely as *fragments of cytoplasm having no function included in the nucleus by chance between the chromosomes as they pass into the resting stage* Although this concept was later abandoned with increased knowledge of nucleolar chromosome rela

tions this is a good description of the formation of the early interphase nuclear bodies we have associated with spindle remnant Similar irregular droplets almost certainly this fraction have been described for several other plant genera for example in *Cassia* (44) *Crocus* (69) and *Narcissus* (85) We have seen spindle remnant in early interphase nuclei in different tissues of *Zea Tradescantia Nothoscordum Lilium* and *Vicia*

In onion cells spindle remnant can be distinguished from nucleolar material as discussed above by fast green and Sudan black staining after chromic acid fixation Also we have found no visible evidence in onion that these characteristic irregular bodies coalesce They rather appear to grow fainter with time and eventually disappear In addition electron micrographs indicate that this fraction at least in early telophase resembles the cytoplasm in its fine structure These observations lead us to conclude that what we have loosely called spindle remnant should not be implicated at least directly in nucleolar formation

It is more difficult to distinguish nucleoli from the fraction we have called 'chromosomal RNA particularly since their fine structures show certain similarities As mentioned above this is probably the fraction distinguished as lamellar pronucleolar substance by Rattenbury and Serra (79) and considered by Estable and Sotelo (30) to be a part of the nucleolonema This fraction seems to us best interpreted as made up of numerous small extra nucleoli arising at a time when competition for necessary precursors is relaxed We have mentioned that numerous extra nucleoli arise for example in enlarged or karyomeric nuclei We feel that there may be a comparable brief period in late anaphase and telophase when the chromosome becomes synthetically active but before the nuclear membrane is formed During this time the extra nucleolus forming loci would not be in competition with the primary nucleolar loci within the restriction of a single nuclear membrane The evidence that such competition does exist in interphase and that the nuclear membrane is a factor in this process is discussed below

II *Competition*

The effect of competition between nucleolar forming sites on nucleolar volume has been emphasized by several authors In hybrids of *Crepis* Navashin (65) showed that a chromosome locus which normally formed a large nucleolus became inactive when it was combined by hybridization with a stronger locus on another chromosome Similarly in *Drosophila* a Y chromosome failed to develop its usual nucleolus when associated with the X in $\widehat{XX}$ males (47) In microspores of maize one chromosome bearing a translocated portion of the nucleolar forming locus formed a large nucleolus in cells bearing the reciprocal

translocation but the same chromosome produced only a small nucleolus in cells containing the normal nucleolar chromosome (61)

Where extra nucleoli form at new chromosomal loci it seems logical to assume that competition is relaxed Extra nucleoli may arise in certain normal cells where as described above nuclei are enlarged or in the process of enlarging Extra nucleoli also arise where nuclei have been fragmented into separate karyomeres This normally occurs in early cleavage stages of certain invertebrate oocytes e g in annelids (56) and grasshoppers (62) and in the divergent spindle mutants of plants (24) The competition between nucleolar sites is thus apparently inhibited by intervening nuclear membrane Where these nuclear fragments do not contain a potential nucleolus forming region as in the irradiated onion or colchicine treated rat liver tissues described above the karyomere does not contain a nucleolus Karyomeres without nucleoli were also reported by Darlington (26) and LaCour (52) who felt contrary to our findings that such fragments were incapable of entering prophase if a nucleolus was lacking

Data on competition are extensive enough to indicate that it is a basic characteristic of nucleolus forming loci In the light of such findings it is logical to conclude that the diffuse nucleolar material arising from many chromosome loci in cells where the normal nucleolar loci have been disrupted involves a similar relaxing of nucleolar competition rather than the failure of an organizer to accumulate chromosome matrix as postulated by McClintock (61)

The decrease in nucleolar number found during liver regeneration we have considered in part the result of fusion but also due in part to competition Here competition appears to be heightened rather than relaxed At 24 hours of regeneration 21 per cent of the tetraploid nuclei contained one large and three minute nucleoli At 36 hours the number of such nuclei dropped to 3 per cent Although it is impossible to determine cytologically the relation between chromosomes and nucleoli in these interphase liver nuclei it is likely that the three nucleoli which we know had been diminishing in size actually disappeared in the interval between 24 and 36 hours Since these cells are tetraploid it would also mean that competition occurred in part between genetically identical loci becoming complete in some cells by 36 hours so that no nucleoli occurred at all on three of the four usual loci

There are two implications of interest in these findings First whether a nucleolus is formed or not at a given potential nucleolar locus apparently depends upon interaction between that locus and its intranuclear environment The environment is conditioned by the availability of precursor material and the ability of other nucleoli to compete for it Second if one nucleolus becomes dominant to the exclusion of others this must make it somehow more effective than smaller nucleoli in obtaining precursors or in forming inhibitors so

that the competition can proceed to the complete suppression of other loci The volume measurements of Bhatia (12) and Parthasarathy (70) indicate that cells with fused nuceoli contain a larger total nucleolar volume than where nucleoli are separate In other words, possibly the larger nucleoli are more efficient in obtaining precursors or are less subject to loss of nucleolar substance through their proportionally smaller surface These observations stress the extreme plasticity of nucleoli which must be in a state of dynamic equilibrium with their immediate intranuclear environment

To carry these speculations one step further if our interpretation of competition is correct, the phenomena of dominance and of increased volume with fusion imply that it is not the locus which competes but the nucleolus itself In others words nucleolar substance appears to contain within itself the ability for continued synthesis of nucleolar material This concept is supported by the studies of Ficq (32) who found incorporation of C^{14} glycine into the RNA and proteins of frog oocyte nucleoli In these cells the nucleoli are known to be detached from the chromosomes and therefore are capable of incorporating precursors without the help of nucleolus forming loci

III *Nucleolar Fusion*

Fusion in bean root interphase is roughly linear with time but liver regeneration data indicate that fusion is not always merely a chance occurrence unrelated to changes in cell function During the first 24 hours a large number of liver nuclei contained nucleoli in the process of fusion At 18 hours of regeneration about 50 per cent of the nucleoli were against the nuclear membrane but in the interval between 18 and 24 hours many nucleoli occupied a central position in the cell and the number of peripheral nucleoli dropped to 22 per cent It seems likely that this movement of nucleoli whatever its cause may aid nucleolar fusion A similar nucleolar movement occurs with changes in synthetic state in neurons of the fish *Lophius* except that the synthetic activity of the cell is in most part directed toward the axon In this case the nucleolus moves to a position near the nuclear membrane toward the axon base (43)

In some tissues as described by Tobias (94) nucleoli characteristically form in telophase already fused into a single body Tobias suggests that synergism of two chromosomes is necessary in such cases for nucleolus formation It is also possible that these cells from telopha e on are engaged in synthetic activity which as in liver regeneration brings about an active fusion of nucleoli

IV *Nucleoli as Gene Products*

Nucleoli form in telophase in intimate association with the chromosomes In some cases the chromatids appear to be pushed apart at the nucleolus forming locus (22 27 71) The nucleolar attachment region in *Chironomus*

shows DNA containing threads fanned out into the nucleolus suggesting that the chromonemata are displaced by the accumulation of nucleolar sub stance (6 7 50) These observations support the suggestion that the nucleolus is the product of the attachment locus and has not formed from the passive coalescence of droplets

Studies on polytene chromosomes have demonstrated several other points of interest The RNA containing structures of the nucleus occur in a spectrum of forms including barely visible to prominent bands puffs Balbiani rings nebennucleoli and nucleoli Although in most cells there is no problem in distinguishing nucleoli from other nuclear structures, in these cells nucleolar substance cannot be distinguished from chromosomal RNA except by arbi trary definitions For example Poulson and Metz (74) consider the Balbiani ring a second nucleolus but find only puffs and no nucleolus at all in the salivary nuclei of *Sciara* Whether the *Chironomus* species studied possesses one nucleo lus or two and whether *Sciara* contains one nucleolus or none depends upon one s classification of these RNA containing components of the nucleus Puffs can also change their morphology during larval development (15) or in response to cold treatment (8)

A similar difficulty is encountered in differentiating chromosomal from non chromosomal material Most authors (e g 37 95) consider nucleoli as distinguishable from chromosomes chiefly on the basis of their lack of DNA In many cells thin Feulgen positive threads traverse the nucleolus or chromatin masses surround it In such cases it is easy to assume that the chromosome enters or surrounds the nucleolus without being a part of it In the polytene nuclei of *Drosophila* and *Chironomus* numerous DNA containing threads enter the nucleolar substance Here it is difficult to distinguish chromo some from nucleolus in the region of attachment but much of the Feulgen negative nucleolus is still distinguishable from the DNA containing chromo some In the case of Balbiani rings and puffs however DNA and RNA containing material become more intimately related and the differentiation between chromosome and chromsomal product is less apparent It is worth emphasizing however that in almost every locus in which RNA occurs the chromsome is noticeably thickened over the bands where no RNA is evident This suggests that RNA is usually if not always associated with extra material presumably protein that results in an expansion of the chromosome Thus while it seems impossible on morphological grounds to distinguish the ribonucleoprotein product from the locus at which it arises these chromosome enlargements suggest that chromosomal RNA at least when identified by the staining technique used here occurs as non chromosomal material In this respect RNA containing bands and puffs are similar to nucleoli

The situation is more complex if we accept the suggestion of Vincent (95) that the 'nucleolonema' may be comparable with a pair of loops, as seen in the lamp brush chromosomes of amphibian oocytes. Ris (80), Callan (18) and Gall (35) have suggested that such loops contain the chromonema (or chromosome thread) too attenuated to show a positive Feulgen reaction for DNA. If so this would imply that the chromosome in addition to forming the usual thread penetrating the nucleolus also winds through the entire nucleolar substance. This concept was supported by Lettre (54) who reported that when chick fibroblast nucleoli were dispersed by the action of adenosine they displayed irregular Feulgen positive threads which he identified as chromonemata. If these threads actually formed an integral part of the nucleolus it is difficult to understand why they would not also appear Feulgen positive in the intact nucleolus. Recently, on the basis of biochemical determinations Monty *et al* (64) have claimed that large amounts of DNA occur in isolated rat liver nucleoli. This interesting observation is in complete contradiction to numerous cytochemical findings. We have been unable to confirm this report (Rasch, unpub.) which we feel is due to contamination from nucleolus associated chromatin and DNA adsorption.

How extensively the chromosome actually contributes structurally to the nucleolus is thus still obscure. In spite of the possible equation of the nucleolonema with the chromonemata particularly by Lettre in most cells nucleoli are readily distinguished from chromosomes by the usual cytochemical methods for RNA and DNA. Also, by implication from our studies on polytene chromosomes other RNA containing structures of the nucleus may probably be considered as chromosome products rather than as elements of chromosome morphology.

The concept that puffs represent active gene loci swollen with the products of gene action, has received support from studies on the variability of these structures. Poulson and Metz (74) described one locus that showed extreme variability in different *Sciara* larvae varying from a single unexpanded band to a large puff. Similar observations have been made on *Rhynchosciara* by Breuer and Pavan (15) who correlated the formation of certain puffs with stages in larval growth. *Chironomus* individuals heterozygous for a puffed locus were described by Beermann (8) in which one homologue lacked the expanded region. Nucleoli may show similar variability in behavior and extra nucleoli can arise in certain stages of growth and later disappear as discussed above. In a few cases nucleoli have also been reported to be 'heterozygous' showing one large and one small nucleolus as in certain *Oenothera* hybrids (11). However the fact that nucleoli are almost universal in every cell engaged in protein synthesis implies that the nucleolar forming locus plays some fundamental role in the process of cell growth.

V Nucleoli as Centers of Protein Synthesis

There have been numerous suggestions in the literature that the nucleolus plays some role in nuclear cytoplasmic interaction (e g 63) Hyden (43) and Caspersson (20) have considered that nucleoli act as mediators between the chromosomes and synthetic processes in the cytoplasm It was suggested that protein materials diffused from the nucleolus to the nuclear margin and in neurons of the fish *Lophius* a gradient in RNA containing material from nucleolus to nuclear membrane was reported Various morphologic changes in nucleoli suggestive of synthetic processes, have been described, including budding -off of nucleolar material into the nucleoplasm and subsequent dissolution (58 63 84) and the formation of fluid filled vacuoles within nucleoli and their extrusion into the nucleus (42 96)

There is a large literature on the reported extrusion of materials from nucleoli through the nuclear membrane into the cytoplasm (see refs 58 and 37 for reviews) Careful observations on such processes are often difficult to make and there are few cases where possible effects of cell damage can be entirely eliminated Also the interpretations of nucleolar extrusion made on the basis of fixed preparations are often, of necessity subjective For these reasons much of the literature on transfer of material from nucleolus to cytoplasm is controversial and difficult to evaluate For example Duryee (29) has described nucleolar extrusion in amphibian oocytes but this has been contested by Callan (17) Nucleolar extrusion in tissue culture cells has been described by Ludford (59) but Lewis (55) felt that such changes were associated with nuclear fragmentation and cell death Prescott (75) finding no uptake of C^{14} uracil into the cytoplasmic RNA of enucleated amoebae concluded that *all* the RNA found within the amoeba cytoplasm and nucleus is elaborated within the nucleus Although this finding is of considerable interest in reality all that it demonstrates is that the nucleus probably plays some necessary part in the synthesis of cytoplasmic RNA This contribution could equally well be through the action of needed enzymes rather than by direct transfer of RNA In some cells at least RNA is almost certainly synthesized in the cytoplasm as indicated by incorporation studies on enucleated *Acetabularia* by Brachet and Szafarz (14) or studies on *Tradescantia* pollen which show rapid cytoplasmic RNA synthesis with no measurable nuclear RNA (92 98) Few authors would quarrel with the concept of Montgomery (63) and others that nucleoli are involved in nuclear cytoplasmic interaction but at present there is little evidence that extrusion of actual nucleolar material in most cells is a normal part of this process

As reported above nucleoli show changes in RNA protein ratios which parallel those in the cytoplasm This strongly suggests that nucleoli like RNA-containing structures outside the nucleus are also centers for protein

synthesis If nucleoli were merely aggregates of ribonucleo protein accumulated by the chromosomes for later transfer to the cytoplasm one would expect RNA protein ratios to remain unaltered during nucleolar growth Instead in both HeLa and liver nucleoli RNA concentration was high in early growth stages and fell later to normal levels Similar patterns have been reported in the formation of cytoplasmic proteins in many cells—for example in hemo globin synthesis during erythrocyte formation (93) A similar shift in RNA protein ratios was found during the meiotic prophase in maize nucleoli by Lin (57) who also concluded that nucleolar RNA was probably utilized in the synthesis of nucleolar proteins

Interpretation of electron micrographs in terms of our fragmentary knowl edge of nucleolar chemistry is at present premature It is probable but by no means established that the RNA together with some protein is located in the fine particulate fraction of nucleoli discussed above It is also probable that the fine filamentous component which at least in some cases (as in Pls IX and X Figs 40 42 45 46) appears distinct from the particles is primarily protein in nature We have determined that areas of *Drosophila* salivary nucleo li that primarily contain the filamentous material are of lower RNA concen tration than the particle containing areas on the periphery The filamentous material that occasionally radiates out from nucleoli usually lacks the par ticulate component We should like to suggest tentatively that the radial com ponent respresents a filamentous protein formed in the nucleolus and extending from it during cell growth In *Amblystoma* pancreas cells filaments of similar dimensions may occasionally be traced through the nuclear membrane and into areas of forming ergastoplasm (Swift unpub) Such filaments might also form the basis for nucleolar movements possibly as a recoil action to their formation

Such concepts are tentative additions to the long list of speculations on nucleolar function already published They are valuable only as temporary frameworks on which to assemble current data and from which to direct future research

SUMMARY

Observations are presented that bear on the problems of nucleolar function in plant and animal cells

1 Three different RNA containing fractions occur in onion nuclei namely the nucleoli spindle remnant and chromosomal ribonucleoprotein Frag ments of spindle material are caught between chromosomes at anaphase forming irregular droplets which disappear in early interphase This spindle remnant can be distinguished from nucleoli because it is negative to certain tests for lipids which stain nucleoli Chromosomal RNA arises as a series

of minute granules in contact with anaphase and late prophase chromosomes In the electron microscope areas of spindle remnant are similar in structure to the cytoplasm while chromosomal RNA resembles the nucleolus in its electron density and the possession of dense particles

2 Two cases have been studied where nuclei have been experimentally fragmented into a number of karyomeres namely onion roots after irradiation with slow neutrons and regenerating liver treated with colchicine In both cases some nuclear fragments contained nucleoli but others did not The total number of nucleoli per cell was greater in karyomeric than in normal cells thus extra nucleoli were formed in association with nuclear fragmentation

3 Extra nucleoli occur normally in the enlarged nuclei of *Tradescantia* lateral bud primordia In these cells growth is inhibited by auxin from the shoot apex

4 Chromosomal RNA as localized by azure B staining has been studied in salivary glands of *D virilis* RNA occurs in association with a variety of regions varying from minute bands to grossly enlarged puffs In *Chironomus* sp the nucleolus is associated with an RNA containing band

5 Two cell types were studied in which growth rates were experimentally altered These were regenerating rat liver and HeLa cells in culture treated with salt solution until starved and then re fed with supplemented medium Both cell types showed changes in nucleolar shape and number associated with particular stages of growth In regenerating rat liver reduction in nucleolar number was due both to increased fusion and to the disappearance of some nucleoli In both cell types nucleoli showed marked changes in RNA protein ratios as determined by microphotometry of tissue sections stained for RNA with azure B and for total protein with the Millon reaction or fast green These changes approximated those found in the cytoplasm

6 Nucleolar number and RNA protein ratios were also determined in bean roots (*Vicia faba*) Reduction in nucleolar number, through fusion was roughly a linear function of interphase time RNA protein ratios remained constant through interphase

7 Two aspects of nucleolar morphology are described as seen with the electron microscope First local variations in RNA and protein concentration can be correlated with differences in distribution and density of RNA containing particulates and with variation in the compactness of nucleolar threadlike structures Second numerous fine filaments about 200 $m\mu$ in diameter are described that extend from nucleoli in some regions into the nucleus

8 From these data we have drawn four conclusions First, the concept that the nucleolus is organized from matrix material in telophase seems untenable Droplets of material interpreted by most authors to be stages of nucleolar aggregation we consider to have no direct connection with nucleolus formation

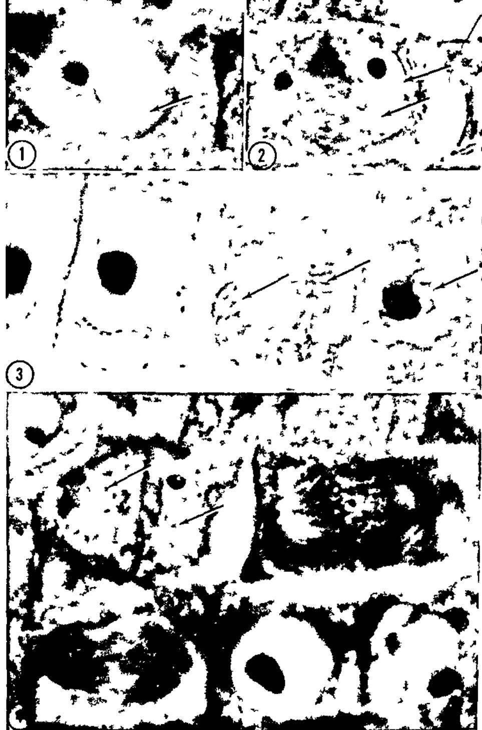
Second, the nucleolus is best considered the synthetic product of a chromosome locus one of a number of forms in which RNA containing materials occur in association with the chromosomes Third nucleolar size and number are influenced by the competition between nucleolus forming sites for certain unknown components in their environment Fourth cytochemical studies on the nucleoli of growing cells support the concept that the nucleolus is a site of protein synthesis possibly of material necessary for the formation of cytoplasmic RNA

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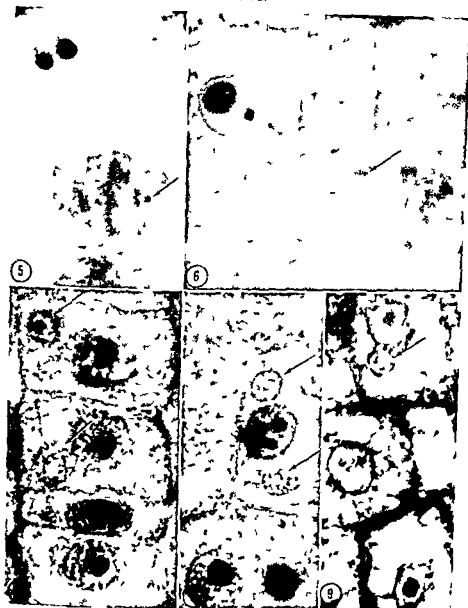
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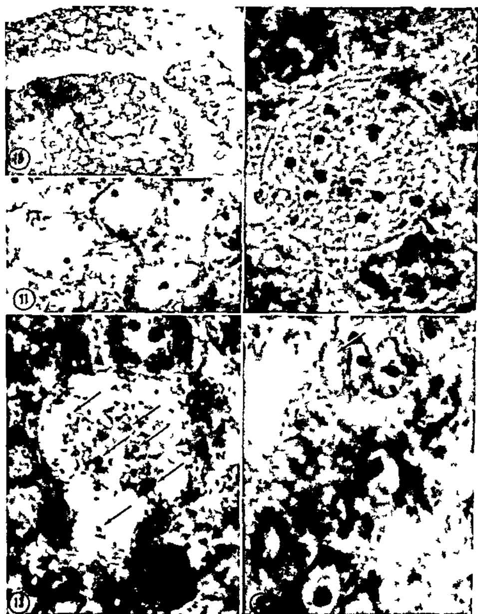


FIGS 1–4 —RNA distribution in onion root tip mitosis All photographs are of material fixed in acetic alcohol treated with desoxyribonuclease and stained with azure B Fig 1 Late prophase with chromosomal RNA visible surrounding chromosomes (*arrow*) Fig 2 Late telophase showing well formed nucleoli and spindle remnant barely visible as a few faintly staining areas (*arrows*) Fig 3 Telophase in center showing chromosomes outlined with RNA-containing material (*arrows*) Prophase at right showing chromosomal RNA In the interphase cells at left the chromatin is completely unstained Fig 4 Mid and late anaphase showing RNA in spindle material Numerous irregular areas of spindle remnant are visible in the late telophase nuclei (*arrows*)

PLATE II

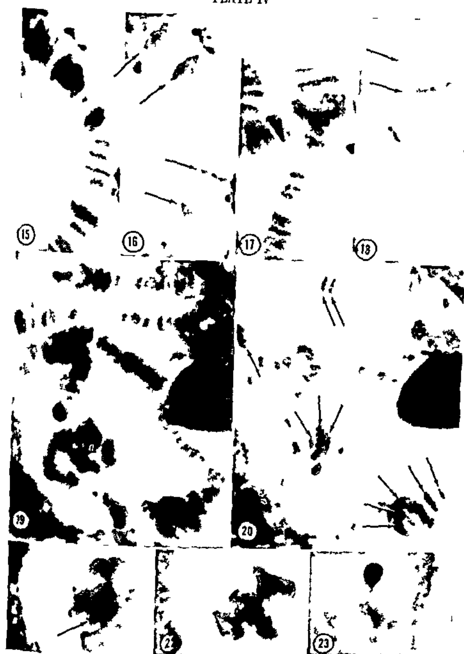


FIGS 5-9.—Figs 5 and 6 Onion roots fixed in Navashin's and stained for nucleoli with fast green. Forming nucleoli are visible in telophase nuclei (*arrows*). Spindle remnant is unstained. Figs 7-9 Onion roots grown from bulbs irradiated with slow neutrons, fixed in Navashin's and stained for nucleoli with fast green (Figs 7, 8) or Sudan black B (Fig 9). Nuclear fragments (karyomeres) are shown by arrows. Some contain nucleoli but others do not.



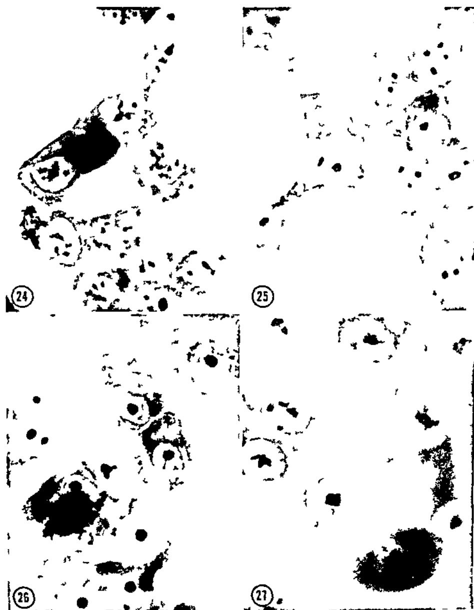
FIGS 10 and 11 —*Tradescantia* lateral bud primordia fixed in acetic alcohol treated with desoxyribonuclease and stained for RNA with azure B Fig 10 shows enlarged nuclei in bud primordium surrounded by two leaf primordia Fig 11 Same field at higher magnification showing numerous small nucleoli in the enlarged nuclei

FIGS 12–14 —Rat liver 48 hours after partial hepatectomy and 24 hours after colchicine treatment stained with azure B Fig 12 Cell with dispersed and partially clumped chromosomes Fig 13 A cell similar to that in Fig 12 but entering interphase Numerous small karyomeres are shown most of which contain nucleoli (*arrows*) Fig 14 Interphase cell with nucleus divided into several karyomeres The karyomere at arrow has no nucleolus

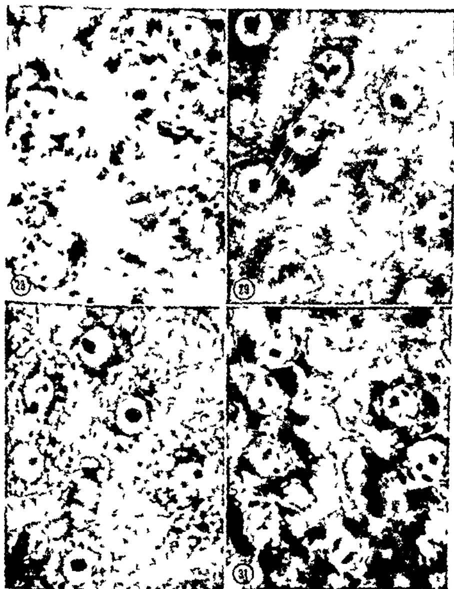


FIGS. 15–20.—Smears of *D. virilis* salivary glands stained with azure B. Figs. 15, 17 and 19 were taken at a wave length of 615 mμ and show the distribution of both RNA and DNA. Figs. 16, 18 and 20 were taken at 505 mμ and show primarily the distribution of RNA only. Principal RNA-containing bands and puffs are indicated by arrows. Note that the lower band in Fig. 17 appears wider than in Fig. 18. This band contains DNA on the upper (distal) edge and RNA on the lower edge. Fig. 20 shows two puff regions, one containing five and the other three adjacent RNA-containing bands.

FIGS. 21–23.—Figs. 21 and 22. The nucleolus attachment area in two sections of *Chironomus* salivary glands showing an RNA-containing band traversing the chromosome in this region. In Fig. 21 the arrow points to a small nucleolus-like body in the adjacent puff. Fig. 23. An bennucleolus from *D. virilis* showing its attachment to an RNA-containing band.



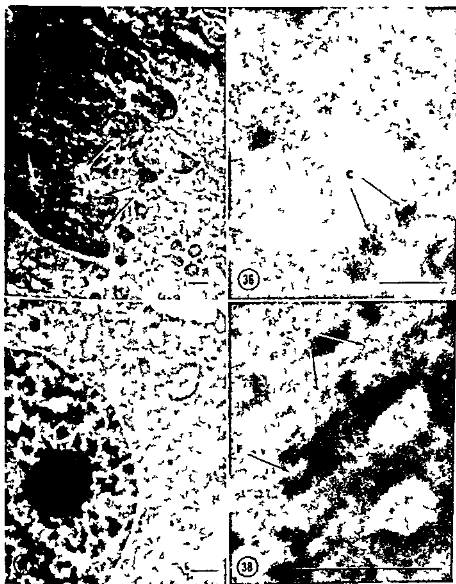
Figs. 24–27.—HeLa cells in cell culture treated with desoxyribonuclease and stained for RNA with azure B. Fig. 24. Control culture in stage of rapid growth. Fig. 25. The same culture after 72 hours in Hank's salt solution. Fig. 26. The same culture 6 hours after addition of complete (Eagle) medium. Fig. 27. The same culture 24 hours after complete medium was added. Note the marked changes in nucleolar size and shape.



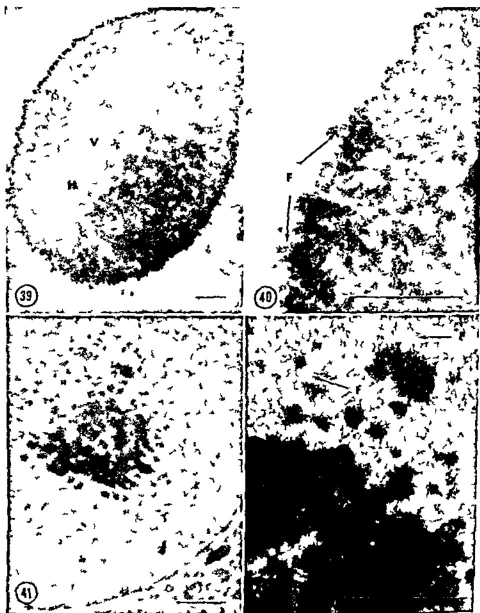
FIGS. 28–31.—Rat liver cells during regeneration following partial hepatectomy treated with desoxyribonuclease and stained for RNA with azure B. Fig. 28. Control liver. Fig. 29. Liver 15 hours after hepatectomy. Fusing nucleoli shown at *F*. Minute nucleoli (apparently newly appearing) shown by arrows. Figs. 30 and 31. Liver 24 and 36 hours after hepatectomy, respectively.



FIGS. 32–34.—Electron micrographs of telophase nuclei from an onion root. Spindle remnant shown at *S* and chromosomal RNA at *C*. Figs. 33 and 34 show enlarged portions of Fig. 32.



Figs 35–38.—Electron micrographs of onion root nuclei Figs 35 and 36 Late anaphase showing spindle remnant (*S*) and chromosomal RNA (*C*) Fig 36 shows a portion of the same cell shown in Fig 35 from another section Fig 37 Portion of an interphase cell showing the nucleolus Fig 38 A portion of the nucleolus from another cell Radiating filamentous material shown by arrows labeled *F* The nucleolus contains a few scattered dense particles similar to those at *C* in Fig 36



Figs. 39 and 40.—Electron micrographs of a nucleolus from *D. virilis* salivary gland. Note small vacuoles (V) and also the homogeneous areas (*H*) and the particulate areas (*P*). In Fig. 40 fine particles appear to line filamentous material (*F*).

Figs. 41 and 42.—Nucleolus from a pancreas cell of *Amblystoma tigrinum*. Filamentous material is shown by arrows.



FIGS 43–46 —Electron micrographs of rat liver cell Fig 43 and 44 show nuclei of normal liver Figs 45 and 46 show nucleoli from tissues 24 hours after partial hepatectomy Nucleoli are less compact show more irregularities in their electron density and contain areas of filaments (*F*) extending into nucleus or to the nuclear membrane

CHAPTER 21

Autoradiographic Studies of the Organization and Mode of Duplication of Chromosomes*

J. Herbert Taylor

By the use of tritiated thymidine for labeling DNA (deoxyribonucleic acid) information has been obtained which allows a reorientation of our thinking about the organization and mode of duplication of chromosomes (27). Chromosomes were found to be composed of two strands or units of DNA which separate and behave in duplication as if they were the two polynucleotide chains of a molecule of DNA of the type proposed by Watson and Crick (28). These experiments were possible because the low energy beta particles emitted by tritium give a resolution in autoradiography that allows one to distinguish the labeled and unlabeled chromosomes within a single nucleus.

EXPERIMENTS ON THE DISTRIBUTION OF LABELED DNA

Tritium labeled thymidine was prepared by exchanging tritium (hydrogen 3) from the carboxyl group of acetic acid to a carbon atom in the pyrimidine ring of thymidine. A product of high specific activity (300 mc/millimole or greater) was obtained and used at a concentration of 2–3 $\mu g/ml$ for growing roots of *Vicia faba* (English broad bean). The root cells have an average division cycle of 24 hours under the conditions of culture. DNA synthesis extends over a period of about 8 hours during middle interphase with about an 8 hour interval between the end of synthesis and metaphase (7). Therefore roots grown for 8 hours in the isotope solution have some cells with chromosomes that have completed their duplication while in the isotope solution. The labeling of chromosomes occurs only during the period of DNA synthesis and only rarely does a cell with labeled chromosomes have time to reach anaphase during the 8 hours in the isotope. Since the thymidine at the concentration

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u ed does not accumulate in the tissue to any significant extent and is utilized almost exclusively for DNA synthesis (15) the labeled precursor pool is quickly depleted when roots are removed from the i otope solution and no component except the DNA is labeled At the end of 8 hours the roots were thoroughly washed and placed in a mineral solution free of labeled thymidine Colchicine at a concentration that would block futher cell divisions but would allow syn thesis of DNA and chromosome replication to proceed was added to this solution Roots fixed after 10 hours in the colchicine solution contained numer ous cells with colchicine blocked metaphases Many of these cells contained chromosomes which were duplicated during the period when the roots contained the maximum amount of the labeled precursor Autoradiographs showed the chromosomes to be labeled rather uniformly from end to end Furthermore the daughter chromosomes which frequently remain tog ther at least at the centromere region were equally labeled

To determine the fate of the DNA that existed in the chromosome before the duplication in the presence of the labeled thymidine these labeled chromo somes had to be followed through another duplication in the absence of labeled precursor This was possible because of the quick depletion of the pool of labeled precursor in the roots when they were removed from the isotope solution Fur thermore the colchicine treatment allowed the recognition of those cells that had duplicated once since the duplication in which they were labeled In these plant cells the colchicine prevents spindle formation and the anaphase separation of chromosomes but after a short delay the length of which appears to be very sensitive to the colchicine concentration the daughter chromosomes fall apart and enter an interphase stage When roots remained in the colchicine solution 34 hours instead of 10 some of the cells containing labeled chromosomes had a chance to advance to the first colchicine blocked metaphase (c metaphase) and then to re enter interphase without a cell division having occurred Duplication of chromosomes occurred and the cells reached a second metaphase under the influence of the colchicine These second c metaphases are readily recognized because the cell now has twice the usual number of chromosomes

An examination of autoradiographs of the tetraploid cells revealed that only half the chromosomes were labeled When the daughter chromo omes remained together at the centromere region one of these could be seen to contain all the lab led DNA while the sister chromosome (chromatid) was completely free of tritium The only exceptions were those daughter pairs in which sister chromatid exchange had occurred i e a break and reciprocal exchange of large labeled segments Indirect evidence had indicated that sister chromatid ex change occurs (22) but this was the first opportunity actually to see the result and be able to determine directly the frequency of such exchanges

A few cells were observed in which a second duplication of chromosomes had occurred during the growth of the roots in the colchicine These could be recognized since they contained four times the usual number of chromosomes Although a complete analysis of all labeled and non labeled chromosomes was not possible in these cells because of the large number of chromosomes and the fact that some of these were lying across each other in the flattened cells enough could be seen to show that the number of labeled chromosomes did not increase when account was taken of the exchanges Only about half the c metaphase chromosomes with two chromatids connected at the centromere in the usual way had a labeled and non labeled chromatid The other half (evidently derived from the non labeled daughters of the first c metaphase chromosomes) did not have either chromatid labeled

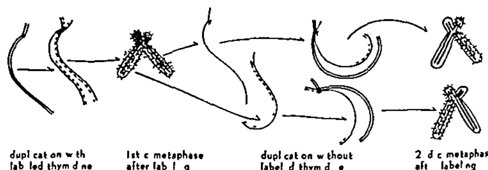


FIG 1 —Diagrammatic representation of the organization and mode of duplication as revealed by autoradiographs The two strands necessary to explain the results are shown although these are not resolved by microscopic examination Solid lines represent non labeled strands and dashed lines represent labeled strands The dots represent grains in the autoradiographs From Taylor *et al* (?)

These observations tell us that the original DNA of the chromosomes is conserved during duplication and except for the occasional sister chromatid exchanges remains in intact units Each chromosome before duplication must contain two of these units or strands Since each of the two daughter chromosomes contains half the new DNA and since they contain equal amount of DNA each must also contain half the original DNA Therefore we must conclude that each chromosome has two units of DNA which extend throughout its length (Fig 1) At duplication these units separate and along each is built a new unit which in our experiments was labeled when duplication occurred in the presence of the tritiated thymidine Each daughter chromosome appeared labeled along its entire length at the division immediately following this labeling But each of these chromosomes carried an original non labeled strand which was so closely associated with the new labeled strand that the whole appeared labeled When the second duplication occurred this time in the absence of the labeled precursor the labeled and non labeled strands separated and each had

a non labeled strand built along it The result was a labeled daughter chromosome (chromatid in the c metaphase chromosomes) and a non labeled daughter chromosome as observed in the autoradiographs

THE NATURE OF THE TWO UNITS OF DNA IN A CHROMOSOME

What are these two units or strands of DNA which we have discovered? Can the chromosome be considered a long single double stranded helix of DNA? The behavior during duplication is certainly analogous to that proposed by Watson and Crick (28) for the replication of DNA The parental DNA double helix was visualized as composed of two complementary polynucleotide chains which unwind during replication Along each of the separating chains would be built its complement from precursors available The sequence of nucleotides in the new chains would be determined by the specific hydrogen bonding required for each new nucleotide to fit along the original chains

The tremendous size differential between these chromosomes and the DNA double helix makes it improbable that a chromosome is a single double helix From the reported DNA content per nucleus an estimate of the length of such a DNA double helix can be made Ogur *et al* (30) reported an amount of 53×10^{-1} gm of DNA per haploid complement of chromosomes in *Lilium*, which has chromosomes of approximately the same size as those of *Vicia* This amount of DNA would make a double helix $1\ 5 \times 10^7\ \mu$ long Since there are 12 chromosomes, each would have to contain a double helix $1\ 0$–$1\ 5 \times 10^5\ \mu$ (1 0–1 5 meters) long The mechanical and energetic problems involved in unwinding such a long double helix are formidable Although Levinthal and Crane (13) have made calculations which indicate that unwinding might be possible if the double helix were not too sharply folded or coiled, the remaining problem of organizing such a ball of yarn into a structure with the dimensions and properties of a chromosome is more difficult

A second possibility that we are dealing with a chromosome made up of numerous DNA double helixes oriented lengthwise along the chromosome axis has to be considered In such a model the DNA double helixes would be organized into two morphological units (chromonemata) perhaps in the form of ribbons as suggested by Taylor *et al* (27) This model fits the classical concept of the chromosome (6 20 23) but if it were accepted the model of replication of DNA proposed by Watson and Crick could not be supported by our data Either the two chromonemata would contain different double helixes or they would in some way share the complementary polynucleotide chains of many double helixes In the first case there would be no necessary separation of the DNA duplex during chromosome duplication and in the latter case the chains would appear to require a stable cross bonding by some substance and frequent interruptions or break points in the DNA double helixes

which would allow separation without mixing of old and new DNA Both models are unsatisfactory for the Watson Crick mode of replication of DNA

The multistranded model of the chromosome could account for the great variation in amounts of DNA found among different species, if the assumption was made that chromosomes of different species might have different numbers of strands per chromosome (20) If the multistranded concept is correct and the number of strands is a species characteristic variations might be expected to occur in the mode of distribution of new and original DNA in different species Since Plaut and Mazia (19) had reported a difference in another species (*Crepis capillaris*) the matter required further investigation They had used thymidine C^{14} to label nuclei in roots in much the same way as reported in our experiments The difference was that the limited resolution obtainable with carbon 14 restricted the analysis to whole groups of anaphase chromosomes rather than individual chromosomes By estimating the number of grains over pairs of telophase nuclei at the first division following incorporation, they arrived at the conclusion that the distribution of the isotope was very unequal in many of the pairs The greatest inequality observed was a two to one ratio of labeled DNA between pairs of daughter nuclei They did not attempt to follow the distribution at subsequent divisions Our results were clearly at variance with these In addition the findings reported by Levinthal (12) were consistent with ours, even though his material—bacteriophage—was phylogenetically far removed from English broad bean He had reported that 40 per cent of phage DNA is contained in one piece which is divided equally in the formation of daughter particles Beyond this initial separation no further distribution occurred during the production of about 150 phage particles that result from the infection of a single bacterium These two experiments with self duplicating structures at opposite ends of the spectrum of living organisms made the doubleness of genetic material appear to be a very fundamental property This concept has been supported by our subsequent work

DUPLICATION OF CHROMOSOMES OF OTHER SPECIES

The next species investigated was *Bellevalia romana* a liliaceous plant from the Mediterranean region (26) Its advantage is that it has only four pairs of relatively large chromosomes in somatic cells three of which have morphologic features that permit recognition at any stage of division at which separate chromosomes can be distinguished Experiments with this plant similar to those with bean roots showed that the labeling of DNA and its distribution in subsequent divisions were exactly the same Both daughter chromosomes were equally labeled at the first division following duplication in the presence of labeled thymidine This condition was observed either when the division

as blocked with colchicine or when the cells were allowed to enter a normal anaphase (Fig 3, *a* and *b*) In Figure 2 are shown the results of counting grains over daughter pairs of chromosomes at the first anaphase after labeling The data are treated in two ways In the first instance the grains over one chromosome selected at random are plotted along the vertical axis and the grains over its sister chromosome at the other end of the anaphase figure are plotted along the horizontal axis For those with equal counts the co ordinates intersect

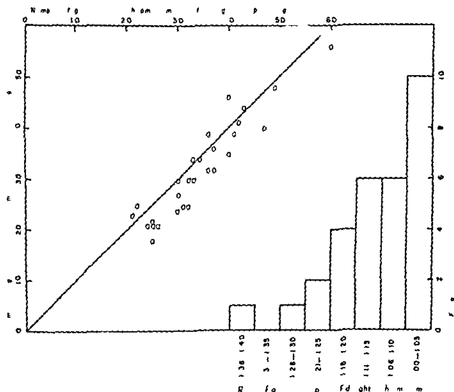
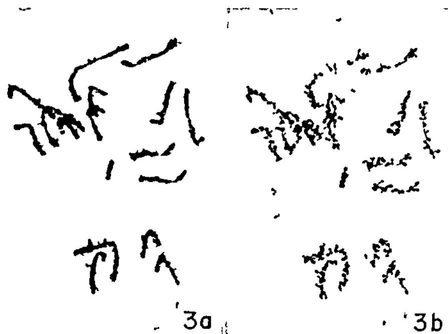


FIG 2 —Graph showing results of counting grains over daughter chromosomes at the first division after labeling occurred

on a line with a slope of 1 All points fall close to this line with none that deviate significantly The pair with the greatest deviation when tested for a 1 1 ratio by chi square yields a *P* value between 0 30 and 0 40 For ease in comparing the variation from equality, the data are also plotted by taking the ratio of the grain counts from each daughter pair and plotting the deviation from unity as a frequency distribution (Fig 2) Since the frequency polygon appears to fit a normal curve there is no reason to believe that the distribution of labeled DNA at the first division is unequal as Plaut and Mazia (19)



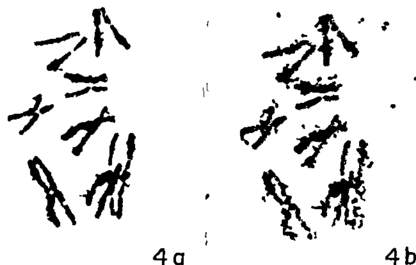


FIG 3 —Anaphase of the first division after labeling in *Bellevalia romana* root tip cell *a* Chromosomes in focus *b* grains in photographic emulsion in focus

FIG 4 —Colchicine blocked diploid metaphase at the second division after labeling *a* Chromosomes in focus *b* grains in focus

contend In addition, the all-or nothing segregation of labeled DNA at the second division demonstrates in an even more convincing way that the original and new DNA are regularly separated in a very precise manner In some experiments roots were placed in the isotope solution for 6–8 hours transferred to mineral solution free of the isotope for 10 hours and finally placed in the colchicine solution for 10 hours These roots yielded a small percentage of diploid cells which showed the second division following labeling (Fig 4 a and b) Because of their low chromosome number these cells are preferred for analysis but unfortunately they do not yield as much information on the origin of all sister chromatid exchanges as do the tetraploid cells

By placing roots in isotope solution for 6–8 hours then in mineral solution containing colchicine for 38 hours autoradiographs could be prepared with tetraploid cells showing the labeled and non labeled chromosomes The chromosomes in these cells had been labeled and had duplicated once more before arriving at the tetraploid c metaphase These cells were satisfactory for the analysis of the frequency of sister chromatid exchanges The largest chromosomes showed about one sister chromatid exchange per chromosome while the smaller chromosomes had a frequency very nearly proportional to their length A large proportion of the exchanges occurred as twins i e two homologous chromosomes in the tetraploid cells frequently had exchanges at the same locus An analysis of the frequency of these twins and the single exchanges indicated that reunion of the two strands of the chromosomal complex was restricted, as if they either had an opposite directional sense or had a mirror image relationship to each other (26) Since the effect of beta radiation emitted by the tritium has not been evaluated the relation between the observed sister chromatid exchanges and the normal frequency cannot be given without further investigation

Finally *Crepis capillaris* was investigated in a series of experiments similar to those performed on *Vicia* and *Bellevalia* and the behavior was the same No evidence supporting the idea of unequal distribution of labeled DNA was found We can only conclude at this point that the doubleness is a fundamental property of genetic material and that it probably indicates the existence of complementary units that duplicate by a mechanism such as that proposed by Watson and Crick Therefore a model for the chromosome is required that will explain how this can be Such a model is possible if we depart from the concept that the DNA is arranged parallel to the long axis of the chromosome

A HYPOTHETICAL CHROMOSOME MODEL

A model suggested by Taylor (25) will serve to explain our results and in addition possesses many intriguing genetic implications (Fig 5) We shall retain the essential ribbon shape suggested by Taylor *et al* (27) but shall assume

that the DNA double helixes are attached to a ribbon shaped central axis or core which is a duplex (a double ribbon). The DNA double helixes are attached in such a way that one polynucleotide chain of each is attached to one ribbon of the duplex and the other polynucleotide chain to the other ribbon. Duplication can be visualized as beginning at the end by separation of the two ribbons of the core. Their separation would result in the separation of the two polynucleotide chains in the region adjacent to their attachment points. This would initiate the unwinding of the double helixes, and replication of the DNA could proceed as proposed by Watson and Crick. With one end of each double helix free to rotate and with single bonds that allow rotation

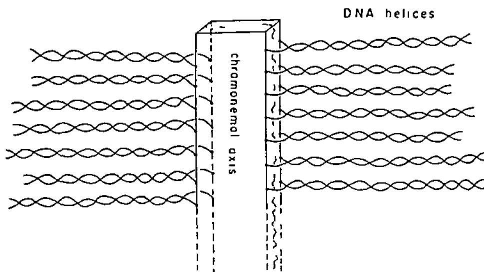


FIG. 5.—Model of a chromosome proposed on the basis of the behavior in distribution of labeled DNA. Side branches represent DNA double helixes. From Taylor (25).

along each polynucleotide chain in regions where separation is occurring the unwinding of relatively short side chains of DNA should present no mechanical problem. As the two ribbons of the core separate we may visualize the assembling of two new ribbons, one along each of the original ones to form two new duplexes. All the new polynucleotide chains would now be attached to the new ribbons and would therefore act as two units in future duplication. Only by exchange of segments along the axis (sister chromatid exchange) would the new units be broken up. The model is merely a skeleton of a chromosome and only enough detail is introduced to explain our data. A number of questions immediately arise concerning the position of the other components believed to be present in chromosomes and the nature of the hypothetical core. Speculations on these matters are probably not fruitful at this time but there is some

evidence that a core actually exists at least in spermatocyte chromosomes (4 17) The electron microscope pictures obtained from thin sections show a laminated structure along the axis of the chromosomes In cross sections the best photographs show two ribbons in each chromatid with DNA distributed peripherally, as predicted by the model

The critical test of the model will probably come from data on genetic recombinations which have always been the most powerful tool for the analysis of chromosome structure However from the beginning these studies have looked to cytology for the material basis and usually the working hypotheses Unfortunately, cytology has not always been able to keep pace with the achievements in the study of genetic recombinations At present the two are far out of balance and a major reorientation of our concepts of chromosome structure is in order The chromosome model proposed here predicts a considerable change in that it adds another dimension for crossing over The conventional type of recombination would involve exchanges along the central axis or core which would resemble mechanically the sister chromatid exchanges observed in the autoradiographs The DNA side chains might represent functional units (Benzer s cistrons) but these functional units should be capable of mutation at many sites and of recombination of the type studied in the fine structure analyses of phage (1 2) Phenomena suggestive of this type of recombination have also been reported in yeast *Neurospora* other fungi and even higher organisms (see review by Mitchell [16]) A test of the model can be made by the study of recombinations in three closely linked loci perhaps equivalent to functional units or side chains Recombinations in any two side chains would be expected to show a strictly linear order of mutable loci but as soon as a third side chain between two rather closely linked ones is carefully mapped with respect to both adjacent side chains discrepancies should appear

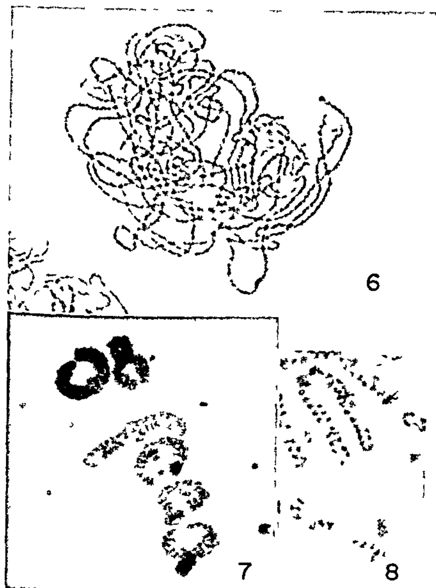
RELATION OF MODEL TO VISIBLE STRUCTURE AND BEHAVIOR OF CHROMOSOMES

The problem that remains is to reconcile the proposed model with the appearance mechanics and behavior of chromosomes as seen with the light microscope Although the rod shaped chromosomes of *Tradescantia* microsporocytes were shown by Baranetzky as early as 1887 to result from the coiling of a longer chromonema this aspect of chromosome structure was given little attention until after 1925 when smear and squash techniques began to be widely used for the study of chromosomes Beginning with the work of Fujii (5) Kaufmann (8) Kuwada (10) and Kuwada and Nakamura (29) the coiled and double coiled structure of metaphase chromosomes was demonstrated in a variety of species with large chromosomes (see reviews by Kaufmann [18] Manton [14] and Cleveland [3]) A great deal of attention was given

to the coiling cycle during mitosis and meiosis The picture that emerged was a coiling cycle that began with a helical coil of small gyre diameter at prophase and changed to one with few gyres and a considerably greater diameter by metaphase (Figs 6 and 7) One view was that the small gyres enlarged and were reduced in number during prophase, while another group described a large coil produced by kinking of the coiled chromonemata of prophase and finally the formation of a fairly regular helix within a helix by metaphase and anaphase

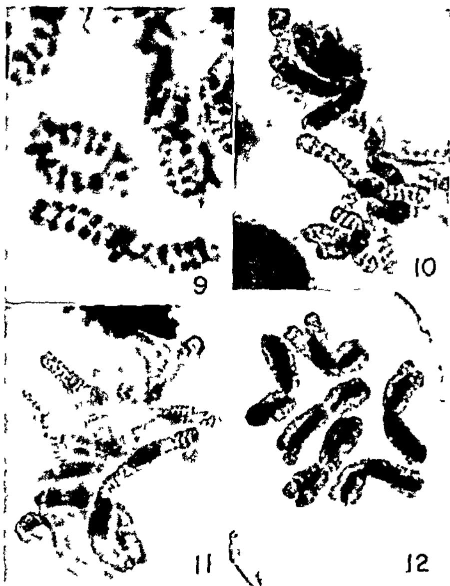
Perhaps both patterns of coiling may actually occur for the two differ only in degree, i e , in the uniformity with which changes in the size of gyres occur If a few gyres enlarge more rapidly than the others the effect would be a *kinking and transition to a condition with major and minor coils* In any case the pattern of coiling of chromosomes is highly variable in different intracellular environments This can be illustrated by comparing meiotic and somatic chromosomes (Figs 6–12) At early meitoic prophase the chromo somes are longer than in any other division stage about 75 μ in early pachytene in *Tradescantia* (Fig 6) However, even at this stage the chromosomes appear to be coiled each having 150-200 gyres in *Tradescantia* (Fig 6 also review by Ris [20]) By metaphase (Fig 7) these long chromosomes are coiled into a major coil of about 10 gyres and are about 12 μ long The coils are reorganized during interphase and at anaphase II (Fig 8) are of a different character There are now 20–24 large gyres per chromosome At the next division—mitosis in the microspore—the number of gyres per chromosome appears to be about the same but the coils are not so easily demonstrated (Fig 12) The chromo somes at all these divisions presumably have two sets of coils, although the smaller set—minor coils—is difficult to demonstrate Minor coils were demon strated in meiotic chromosomes in the original publications of Fujii (5) and Kuwada (10) and later by many investigators Ruch (21) has more recently criticized these interpretations and presented evidence that chromomeres are not actually coils However when two rod shaped chromonemata fit together to form a single rod circular in optical section (Figs 9 and 10) they are best interpreted as meshed helixes

By altering the environment, the chromosomes may be caused to coil differently in both meiotic and somatic cells In Figure 9 are shown the meiotic metaphase chromosomes of *Tradescantia* as altered by growing the anthers in culture after separation from the plant (24) Figures 10 and 11 show altered chromosomes from tapetal cells which are also from the cultured anthers One difference between these chromosomes and those in more typical somatic divisions is the close association of two chromatids which remain together until anaphase This behavior is of cour e usual for the first meiotic division in *Tradescantia* (Figs 7 and 9) The condition is also found in a few



FIGS 6–8.—Fig 6 chromosomes at meiotic prophase in *Tradescantia* early pachytene From Taylor (24) ×3200 Fig 7 meiotic metaphase I chromosomes of *Tradescantia* fixed in acetic alcohol and stained with aceto-orcein ×1400 Fig 8 meiotic anaphase II chromosomes of *Tradescantia* pretreated with 0.01 per cent KCN fixed and stained in aceto-orcein ×2230

tapetal cells from anthers fixed directly after removal from the plant but is more frequent in the cultured material. The chromonemata of the two chromatids appear to be closely associated within both major and minor coils in contrast to the usual situation in somatic cells, where chromatids are separated from rather early prophase and sometimes from the earliest recognizable pro



FIGS 9-12.—Fig 9 meiotic metaphase I of *Tradescantia* from cultured anthers fixed and stained in aceto-orcein ×2230 Figs 10-11 mitotic metaphases in tapetal cells of *Tradescantia* fixed and stained in aceto-orcein ×2230 Fig 12 mitotic metaphase in the microspore of *Tradescantia* fixed and stained in aceto-orcein

phase stages This situation serves to emphasize the difference in timing between duplication and separation of chromosomes for there is every indication that the chromosomes in these cells are duplicated long before they separate (25)

The doubleness in size of the chromonemata due to the late separation may account in part for the larger coils in meiosis and these peculiar somatic metaphases but considerable variation occurs as may be seen by comparing Figures 9 10 and 11 all of which have the chromonemata of two chromatids closely associated Although these illustrations emphasize the variations that may occur in coiling chromosomes nevertheless show a high degree of uniformity in their coiling patterns when similar cells at the same stage in the life cycle are compared in similar environments

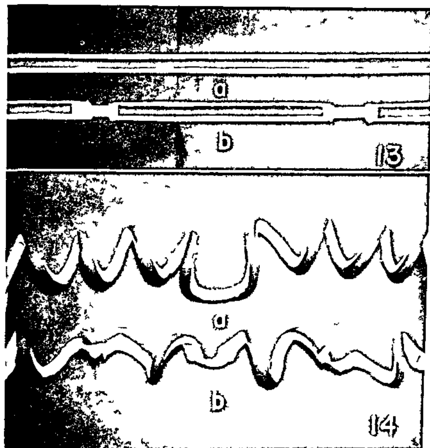
Apparently the size and pattern of minor coils in chromosomes are rather constant since the chromomeres have been mapped in several species and the number and sequence of the morphological differentiations are reproducible Probably the best demonstration of the morphological variations along the chromosome which result in the formation of reproducible coils is the banding pattern in the giant chromosomes of the salivary glands of the Diptera Here we have the best view that we ever get of the longitudinal variation since the giant chromosomes appear to be bundles of completely extended chromonemata However there cannot be a one-to-one relationship between bands and meiotic chromomeres Each chromomere must represent several bands

A variety of mechanisms has been proposed to explain coiling but the most productive one was that of Kuwada (11) who visualized the formation of a series of twists in the chromonemata which were later translated into gyres of a coil The unsolved problem was the origin of the twists that would produce the regular coiling and yet explain the variations observed

Another point concerns the direction of the coiling of the chromosomes A series of investigations (see reviews by Nebel [18] Kaufmann [8] and Manton [14]) have demonstrated that the direction of coiling is not a fixed property of a chromosome segment Coiling is essentially random although there is a tendency for the coils to be in one direction in a chromosome arm

As pointed out by Taylor *et al* (27) a ribbon with edges and middle that contract at different rates will roll or fold into a trough shaped structure and at the same time assume the form of a helix (Figs 13 *a* and 14 *a*) In the model shown the edges shrink faster than the middle but the model works equally well when the middle shrinks faster To produce a coil with a gyre diameter about three times the diameter of the rolled ribbon requires only that the center contract about twice as fast as the edges In the side-chain model if we suppose the DNA double helixes to be attached so that they are adjacent to each other the amount of shrinkage of the edges of the ribbon

is definitely limited while the central strands would be free to shrink further Such differential shrinkage would give the model its tendency to coil One difficulty of a model with a continuous stiffening is that the number of right and left turns has to be equal in each chromosome unless one supposes that the ends rotate Since equality is not observed and rotation of ends would



FIGS 13 14 —Model to show how a ribbon will coil into a helix when the edges contract faster than the middle Fig 13 *a* a stiff mid rib of nylon is sewed down the middle of a strip of elastic while it is stretched Fig 14 *a* when allowed to contract with the ends not allowed to rotate the structure assumes the form of a helix with an equal number of right and left gyres Fig 13 *b* model with interruptions in the mid rib Fig 14 *b* this second model tends to form alternating gyres right and left for each coiling segment

make a plectonemic coil the separation of which would be difficult to explain it appears best to assume that rotation of ends is restricted to a few turns at most, which would produce the observed relational coiling

Other requirements are that chromosomes form chromomeres and that additional sets of coils of larger gyre diameter be accounted for If we make the

assumption that certain regions of the core have relatively few side chains as suggested by the variations in DNA concentration accompanying the banding of the dipteran salivary gland chromosomes the provision for the chromomeres is made As the core shrinks the regions with many side chains will coil first and the regions with few or no side chains will absorb the residual twists that are produced by coiling of the other segments (Figs 13 *b* and 14 *b*) For each gyre formed a compensating twist in the opposite direction must occur There will be a tendency for adjacent small coiling segments (chromomeres) to compensate for the twist of each other by coiling in opposite directions As coiling proceeds from any segment there will be a strong tendency for segments to alternate in direction of coiling The direction of coiling of the first segment will be random at each coiling cycle but the number of coils per segment will be relatively constant under similar environmental conditions The condition necessary to produce a certain fixed number of residual twists would be that the alternating segments form different numbers of gyres as would be the case if they were of different lengths The result could be that the number of right and left gyres would not be equal in a given chromosome The net difference in number of gyres in the two directions which would be a constant feature of a chromosome would result in a regular number of residual twists which would be absorbed by the interchromomeric regions Later with further contraction of these regions the twists would be translated into a second and perhaps even a third set of coils in long chromosomes

The model accounts (1) for the built in twists required by Kuwada s mechanism of coiling (2) for chromomeres and the regularity in numbers of coils and (3) for the randomness in the direction of coils Further it would provide for the observed variations in coiling in different tissues and under different environmental conditions if we only make the assumption that the cellular environment affects the rate and or degree of contraction

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CHAPTER 22

Structure of the Mitotic Spindle*

Raymond E Zirkle

THE mitotic spindle is an essential part of the machinery that makes possible the orderly distribution of chromosomes from a mother cell to the two daughter cells It apparently has nothing to do with the replication of the chromosomes a process that takes place considerably earlier than the formation of the spindle Having made this brief reference to spindle function I shall say little more about it we are here concerned primarily with structure

As observed with the light microscope the spindle is a transitory organelle that appears rapidly (usually in a matter of minutes) and likewise disappears rapidly all of this occurring in close temporal relations with other events in the cell In molecular terms it is an ordered structure that is assembled quickly from submicroscopic components and is also disassembled quickly in an orderly way Thus aside from its intrinsic importance as part of the mitotic machine the spindle may have a more general significance for that part of molecular biology which deals with the problem of construction of cell organelles of microscopic and submicroscopic size from macromolecules that are smaller by from two to four orders of magnitude

Ever since the discovery of the spindle its makeup has been largely a mystery Recently there has been a quickening of research on its structure two general approaches being used (1) mass isolation of spindles and subsequent physical and chemical analysis of their contents (2) intensified study of the individual spindle *in situ* in the cell and particularly in the living the chief techniques being (*a*) improved optical methods (*b*) various methods of taking the spindle apart or of inhibiting its formation and (*c*) improved micromanipulation

Now in discussing this resurgence of spindle investigation we should note what types of spindles have actually been used Naturally each investi

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gator uses the material that is accessible to his particular technique Among the very wide variety of cells in which spindles have been observed microscopically observers have long recognized certain structural differences that may be important (*a*) One is the presence or absence of asters In some kinds of cells there are very large asters located characteristically at the ends of the spindle On the other hand other cells have small asters and still others have none *at all that can be seen with present methods of observation* (*b*) Then in certain cells we have the oddity of monopolar spindles instead of the normal bipolar ones The classic example of this is the famous division in *Sciara* where only a half spindle is present (or at least only one pole of a spindle can be seen) (*c*) Then there seem to be among various kinds of cells very wide differences in the relations between the appearance of the spindle and the disappearance of the nuclear membrane In most animal cells the membrane is pretty well gone before the spindle appears but there are some cells particularly among Protozoa in which the nuclear membrane never disappears The spindle forms and the mitosis takes place *inside* the membrane In general among the Protozoa and the lower plants there are many unfamiliar types of mitosis that despite their oddity apparently go through the mechanics of chromosome distribution without many mistakes

What sorts of cells have been used in this recent work on spindle structure? First there are certain marine eggs For many years these have been chosen for mitotic studies of various sorts the favorites being the ova of such species as sea urchins sand dollars and marine worms Another preparation that has been used quite frequently is the grasshopper neuroblast in surviving culture Finally many studies have been made on vertebrate cells in culture particularly from chick and amphibians the amphibian cells in particular show many of the mitotic details very clearly in the living In all these cells the spindles are normally bipolar and they appear after the disappearance of the nuclear membrane They have asters of various prominence in the marine *eggs very large in the grasshopper neuroblast* less so in the vertebrate cells even less In this group of biological objects we certainly do not have representatives of all variants of spindle structure Thus making due note of the fact that we are perforce dealing with a limited variety of experimental material I shall now discuss spindle structure under the following heads first the properties of the completely formed metaphase spindle second the mode of its formation third its natural disappearance in the mitotic cycle and fourth its artificial destruction or diminution

PROPERTIES OF THE METAPHASE SPINDLE

The early students of mitosis working with *fixed* cells and the light microscope frequently observed that the metaphase spindle contained longitudinal

"fibers" (1). Many regarded these as artifacts of fixation; others regarded them as "real," i.e., truly present in the living; and some classified them as "continuous" (extending from pole to pole) and "chromosomal" (extending only half as far, i.e., from the poles to the kinetochores on the spindle equator). Since the electron microscope has become available, this filamentary structure in fixed and "stained" material has again been observed (e.g., 5, 6).

In *living* cells the general recognition of longitudinally oriented components was long delayed because in the light microscope with ordinary transmitted illumination or even with phase contrast optics the spindle usually can be visualized only in silhouette, as a relatively granule free region in the granule rich cytoplasm (Fig. 1). Although some microscopists noted that, under certain conditions, e.g., with the cell in an acid medium, longitudinal striations were



Fig. 1.—Newt tissue culture cells in metaphase illustrating limited visualization of spindles by ordinary light microscopy. In each cell the spindle is the relatively clear granule free region lying along the diagonal from upper left to lower right. In *a* the visualization is much better than average; the left end of the spindle is accurately located by the centriole (arrow). In *b* the visualization is about average as seen in this type of preparation. Medium dark phase-contrast. Size of each photographed area about 40 × 60 μ. From Bloom *et al.* (11).

to be seen in the spindle, the objection was commonly raised that these were not true living spindles but abnormal ones produced by the special conditions (1). However, during the last two decades the presence of longitudinally ordered components in the living spindle has been convincingly demonstrated by two widely diverse approaches.

First, with successive improvements in polarization microscopy by such investigators as Schmidt (2) and Inoué (3), a wide variety of living spindles has been shown to be birefringent (e.g., Figs. 3 *d*, 4 *a*). The double refraction is generally positive with respect to the long axis of the spindle and is such as would be produced by an approximately parallel arrangement of elongate submicroscopic components. The second approach involves micromanipulation. In the grasshopper neuroblast Carlson (10) observed that when the spindle was pushed with the microneedle it behaved like a semisolid body in a much more fluid matrix (cytoplasm). If the microneedle was inserted partway into the spindle near the equator and no external force was kept on the needle, the latter was expelled from the spindle, always at the end, never at the side.

When an external force was applied to the needle in the direction of the spindle's long axis, the expulsion was speeded up but when the force was applied laterally the entire spindle was shoved ahead of the needle, and the latter was not expelled These observations not only reinforce the evidence gained with the polarization microscope that the main structural components of the living spindle are elongate and lie more or less parallel to the spindle's long axis but also indicate that they have *considerable strength in that direction* where is their cross bonding is relatively weak

During the last decade some basic data on the chemical composition of the spindle have been obtained *The water content is very nearly equal to that* of the adjacent cytoplasm This follows from the regular observation by numerous observers using phase microscopy, that the living spindle *in situ* has a refractive index so close to that of the cytoplasm that the two regions can be distinguished only by their relative content of granules In the sea urchin spindle Mitchison and Swann (4) estimated, by means of the interference microscope, the absolute value of the water content to be 84 per cent

The remaining 16 per cent—the dry matter—is naturally of pre eminent interest Particularly noteworthy in this respect are the investigations of Mazia and his collaborators on mass isolated material from synchronously dividing sea urchin eggs (5 7, 8, 9) The morphological fraction isolated from each cell is designated by Mazia as the "mitotic apparatus" and consists of the spindle the prominent asters and the chromosomes The chemical analyses show the dry matter to consist of at least 95 per cent protein 2 or 3 per cent nucleotides (or at any rate material strongly absorbing at 260 mμ), and a very small amount of DNA presumably from the chromosomes, which in the sea urchin egg represent a very small fraction of the total mass of the mitotic apparatus The great preponderance of the protein of course suggests that this is the major if not the sole structural component of the spindle (and asters) Moreover since electrophoretic analysis of the protein (7) showed it to consist of only two components with one strongly predominating, it is easy to picture the spindle as built up primarily of molecules of only one type of protein Since the molecular weight of the predominantly abundant protein was determined to be about 45 000 it is necessary to account for the approximately parallel array of spindle components indicated by the birefringence that these small protein molecules be linked together From consideration of the chemical agents that stabilize and solubilize the mitotic apparatus Mazia has suggested a model for such linkage through S S bonds (8)

MODE OF FORMATION OF THE SPINDLE

Let us now discuss some recent information about the formation of the spindle Microscopic observation of this process has been rare, but in newt

cells it is slow enough that we have been able in several instances to obtain serial photographs of the same spindle at various stages of its growth. In Figure 2 are shown four frames of a motion picture of a cell that is unique among the thousands of mitoses that, for various purposes, we have recorded by phase contrast cinematography (11). The uniqueness consists in the presence of numerous tiny granules or droplets that, displaying a strong affinity for centrosomes and developing spindle, mark fairly accurately the locations and boundaries of these structures. It is seen that as the two daughter centrosomes move apart in prophase the spindle forms between them. In the motion picture this process is revealed as a very orderly one. In fact, the increase

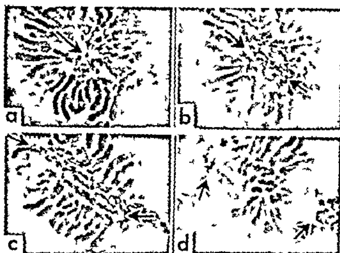


FIG 2.—Growth of spindle as observed by phase-contrast microscopy in a newt tissue culture cell. *a* Late prophase. Centrosome (or the two closely juxtaposed daughter centrosomes) surrounded by numerous granules or droplets. *b* Daughter centrosomes moving apart, the spindle forming between them, spindle and centrosomes outlined by granules. *c* Centrosomes have just ceased to move apart and spindle has accordingly achieved maximal length. Both spindle and centrosomes still outlined by granules. *d* Spindle no longer surrounded by granules, all of which are now arranged about the centrosomes. Chromosomes have taken metaphase position on equator of spindle. Photographic data same as Fig 1.

in length is linear with time, the lengthening ceases suddenly. Whether these apparently simple kinetics are of any basic significance must await further analysis.

The unique cell just described was photographed early in 1952. Since then we have watched for others containing the tiny marker granules but have found none. However, in the meantime Dr Robert Uretz has equipped the laboratory with a polarization microscope which makes visualization of living spindles a fairly routine matter. With this apparatus he and Dr Edwin Taylor have repeatedly confirmed the story of spindle formation outlined above

Stages of elongation are shown in Figure 3 Dr Taylor (12) collected data on some dozen individual spindles, taking motion pictures of some and regularly observed the linear rate of elongation (mean, 1 43 $\pm$ 5 7μ/min) to maximal length (mean 34 0 $\pm$ 5 7 μ)

The orderly elongation of the spindle, its birefringence at all stages of its growth, and the afore mentioned data from Mazia s laboratory on its chemical constitution taken together make it easy to picture Mazia s protein molecules

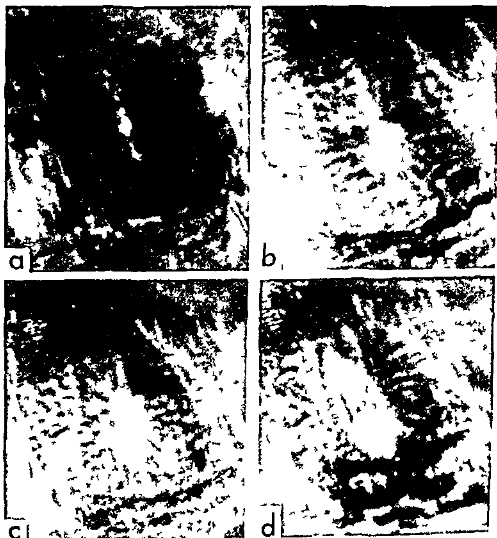


Fig 3 —Formation of spindle in a newt tissue culture cell as seen with the polarization microscope In addition to the spindle certain other parts of the cell show birefringence most of them because of orientation due to strain The chromosomes are seen as faint black rods Area photographed 55 × 70 μ Photographs by R B Uretz

being strung together, one at a time into roughly parallel longitudinal elements If one likes this picture (and, of course it may not be correct) some fascinating questions arise For instance what is the function of the centrosomes? Are they perhaps machines which link the protein molecules together, the resulting strings feeding out between them and pushing them apart? Again what limits the process? Does it proceed until all the building blocks are used up thus accounting for the sudden cessation of elongation? Such questions, as well as the obvious one concerning the extent to which the somatic cell spindle of the newt typifies those of all cells are for future investigation

On the other hand certain other questions have been not only asked but at least partially answered One very old one is Where does the spindle material come from nucleus or cytoplasm? Or in terms of our current picture where does Mazia s protein come from? For the sea urchin egg Mazia and Roslansky (13) have supplied a pretty convincing answer Thus in the first cleavage of the egg of *Strongylocentrotus purpuratus* a single isolated mitotic apparatus contains about 7×10^{-9} gm of protein In the nucleus on the other hand if one uses Mitchison and Swann s value (4) for protein content it is readily calculated that there cannot be more than $0\ 15 \times 10^{-9}$ gm of protein Accordingly in this cell at least it appears that most if not all of the spindle protein must come from the cytoplasm [1]

Of course the foregoing argument about nucleus versus cytoplasm involves the implicit assumption that the spindle protein is already present in the cell a protein and that spindle formation involves essentially the assembly of the protein molecules into filaments There is however the possible alternative that only the building blocks (amino acids and or peptides presumably) are present before the spindle starts to form and that spindle formation involves the synthesis of protein molecules as well as their arrangement into strings Evidence bearing on this question has been obtained by Mazia and Kane (9 p 180) and by Taylor (12) The Mazia group starting with the knowledge that the protein in the isolated mitotic apparatus was almost quantitatively precipitable at pH 4 5 found that a large amount of similarly precipitable protein could be extracted from unfertilized sea urchin eggs Since under the conditions of this extraction the apparatus was insoluble they then extracted samples of fertilized eggs in various stages of mitosis and subjected all protein samples, including that from the unfertilized eggs to identical ultracentrifugal analysis The sedimentation pattern of the protein from the unfertilized egg showed two peaks one of which (corresponding to the lighter component) was absent in mitotic stages when the mitotic apparatus was present and reappeared after it had disappeared This would strongly indicate but of course

1 See [illegible] contrary evidence presented by Carlson and Gaulden (1?) that in the grasshopper neuroblast the spindle is formed from nuclear material (karyolymph)

not prove that the spindle is formed from extractable protein present in the cell before mitosis begins

Taylor's approach (12) was quite different He applied chloramphenicol an inhibitor of protein synthesis, to newt tissue culture cells in various early mitotic stages Using the polarization microscope to visualize spindles, he found that in cells exposed to the drug from 0 to 15 minutes before the first birefringence could be seen (Fig 3 *a*) the spindles developed to substantially normal length and anaphase was normal When the drug was applied from 15 to 30 minutes before detectable start of spindle formation the spindles attained progressively shorter length, the anaphases nevertheless being normal and if it was applied still earlier, the spindles (and/or asters) never progressed beyond the incipient stage (Fig 3, *a*) and no normal anaphase ensued [2] Probably the simplest interpretation of these results is that the bulk of the spindle protein is synthesized in a 15 minute period starting about 30 minutes before spindle assembly begins (accordingly in late prophase) Thus, although the *time of synthesis in the newt cell would seem to be later in the mitotic cycle* than in the sea urchin egg, the main conclusion is the same in the two cases e g , all the protein used in building the spindle is synthesized before assembly starts

NATURAL DISAPPEARANCE OF THE SPINDLE

In anaphase as the two groups of daughter chromosomes separate the total pole to pole length of the spindle increases It was early recognized, from fixed and stained preparations, that the spindle mid region between the two chromosome groups was not of the same nature as the residual regions between chromosome groups and poles This mid region was commonly designated the interzone (1) With the polarization microscope this structural differentiation is readily observed in the living cell Swann (15) showed in the egg of the sea urchin *Psammechinus miliaris*, that as the chromosome groups move apart in anaphase, the spindle region between them ('interzone) decreases greatly in birefringence, and so does a small region just ahead of each chromosome group This orderly disappearance of the birefringence has also been observed by Uretz in the sand dollar egg, and the conclusion that it indicates *breakdown of the spindle structure is in agreement* with Carlson s observation (10) that these regions of decreased birefringence behaved toward his micro needle as if the longitudinally oriented structure were almost entirely gone We thus get a glimpse of normal spindle disappearance as a methodical and spatially progressive process This is not surprising since we have seen that spindle formation likewise has such characteristics

2 Some of these cell divided by the process of false anaphase described by *Bloom et al* (11) and by Zirkle *et al* (14)

ARTIFICIAL DESTRUCTION OR DIMINUTION OF THE SPINDLE

Spindles have been destroyed or diminished in various ways but here I shall discuss only certain experiments in which the kinetics of the process could be observed to some extent

Many chemicals added to the cell medium destroy spindles or inhibit their formation Colchicine especially has been used thus on a wide variety of cells (16) In our present context the work of Carlson and Gaulden (17) is particularly interesting By adjusting the colchicine concentration they were able either to destroy the spindle in the grasshopper neuroblast or to reduce its size and in some instances they could observe the progressive diminution in length and diameter of individual spindles Whenever a spindle was destroyed there appeared in the cell one or more foreign bodies that they called hyaline globules if a spindle was only partially destroyed the volume of the hyaline globule(s) was greater the smaller the spindle became when the spindle could be observed in progressive diminution the hyaline globule(s) became progressively larger Thus it would appear that in this cell type a large fraction of the disoriented spindle substance (protein?) precipitates as droplets in the cytoplasm

In the same cell preparation spindles can also be destroyed or diminished by ultraviolet irradiation Carlson and Hollaender (18) first accomplished this with 2250 A radiation and Carlson (19) later reported that wave lengths 2250 and 2804 A are very effective 2400 A less effective and 2537 2650 and 2967 A only slightly effective in inducing the formation of the hyaline globule This action spectrum would appear to resemble the absorption spectrum of a protein

Is this the spindle protein Somewhat surprisingly the answer is quite likely negative In the course of our studies on irradiation of small parts of newt tissue culture cells Drs Bloom and Uretz and I (11 14) have found that ultraviolet microbeams of various wave lengths from 2250 to 3000 all readily destroy or diminish spindles but it is not necessary to irradiate the spindle itself Bombardment of a small portion of the cytoplasm suffices (Fig 3) This ultraviolet action is clearly an indirect one We have tentatively explained it (11 14) in terms of a spindle poison formed by ultraviolet photochemistry from some cytoplasmic constituent

In our newt cells when the ultraviolet dose is properly adjusted the birefringent spindle (Fig 4 a) disappears slowly enough that one can see that the process is essentially the reverse of spindle formation (Fig 3) The spindle does not suddenly disappear *in toto* on the contrary the process is orderly and progressive just as observed by Carlson and Gaulden (17) when they exposed grasshopper neuroblasts to colchicine

Chairman's closing remarks

We can all agree that the first part of this Symposium has fully lived up to the expectations to which it had given rise and that it has amply justified the efforts of our organizer Dr Chester Jones, and those of Prof Pumphrey at whose invitation we are here We are deeply grateful to both of them As I anticipated our discussions have also proved to have had a broader biological base than almost any previous symposium that has dealt with endocrinology Those who have contributed have spoken with authority and no one present can fail to have been impressed both by the interest of the narrative and by the immense amount that is already known about the reproductive processes of vertebrates Research workers on mammals and birds have been regaled by the knowledge of others who study fishes and lizards and I hope the reverse also applies Dr Hoar s paper has shown us a fascinating new world of endocrinological mechanisms in fish Prof Kehl has proved that the small band of workers who study reproductive mechanisms of reptiles yield nothing in interest to those who work on mammals and the same is true of the Amphibia as demonstrated so convincingly by Prof Gallien and Dr Smith In their turn Dr Harrison Matthews and Prof Amoroso have shown that the theme which gives this rich array of facts its enduring interest and which binds them into an understandable and continuous pattern is primarily biological as opposed to chemical in its impact The story to which we have been listening finds a ready place in the field of evolutionary discussion

The bringing together of all this information has at the same time revealed how extensive are the gaps that remain in our knowledge This could not have been better illustrated than it was by Dr Dodd who told us that while there are 20 000 species of teleost only fifty of them belonging to ten different orders have ever been studied What has also become apparent is that reproductive organs secondary sexual characters and patterns of reproductive behaviour not only differ in an almost infinite way throughout the vertebrate series but that the physiological and biochemical mechanisms of the reproductive system also vary considerably

Two features do however stand out as constant in our varying pattern the hypophysis and the gonads Dr Dodd took issue with the suggestion that ascidians possess an organ which could be regarded as a hypophysis But that does not affect the generalization that the pituitary gland occurs throughout the vertebrate series Similarly the gonads have been shown to be homologous structures even in those cases where they alternate between being testes and ovaries not only in structure but also in endocrine function—regardless of the fact that the corpus luteum may be a structure of somewhat dubious significance in lower vertebrates It is not surprising therefore that a recent remark of Prof Medawar should have been echoed here—that evolution in the field with which we are concerned consists of changes of hormonal emphasis rather than of kind or to quote him verbatim of evolution of a new mode of tissue response to hormones of a category already in being

If we assume the chemical identity of hormonal preparations that have the same effects in the same kind of test this generalization could hardly be better illustrated than it has been by Prof Witschi s showing that apparently pure mammalian FSH may have a very potent melanophore-expanding effect in Amphibia FSH is thus MEH and no doubt the contrary proposition also applies In his turn Dr Leathem has drawn our attention to critical specific differences in the way given hormones act One cannot but wonder whether the taxonomic variations in hormonal response that he and Prof Gallien have exposed in different groups of vertebrates are any less striking than those revealed by the skins and skeletons in a Natural History Museum One might even ask whether variations in the glycogen content of accessory reproductive organs do not constitute a basis for taxonomic differentiation (*pace* Dr Mann)

But caution is also needed As I remarked when introducing my own and Dr Eckstein s paper the variety that can be seen in the sexual cycles of even the single order of mammals is so great that firm classification becomes extremely difficult Dr Parkes took issue with me on this point I think however that we were at cross purposes for I am in full agreement with all he said However much reproductive mechanisms vary we must at least try to fit them into a pattern for knowledge can advance little if we do not generalize My diffidence was not directed to this point but to the prevalent idea that the way we classify reproductive patterns and conceive of their evolution represents more than a working hypothesis The sequence of change which can be imagined when reproductive or endocrinological processes are compared provides a scheme by which to remember and understand' diverse facts but it does not necessarily have any connexion with the actual phases in the evolution of the mechanisms we are considering nor with phases in the evolution of animals which manifest them I cannot help wondering therefore whether our story is an evolutionary one in the accepted sense of the term or whether it merely seems so because the amount of information we have increases progressively as we pass from fish to mammals

Certain evolutionary inferences do of course seem soundly established Some have been referred to by Dr Harrison Matthews and Prof Amoroso in their discussion of the evolution of viviparity We have also had developed for us a picture of the gradual and evolutionary capture of the gonads by the pituitary—and now according to Dr Jost of the possibility of the recapitulation of this capture during embryogenesis But the picture is nevertheless a patchy one We have been told that practically nothing is known about the interrelationship of the pituitary and gonads in cyclostomes What we know about the elasmobranchs provides only the hazy outlines of a picture The design can be better seen when we get to teleosts and amphibians But the full pattern of pituitary-gonadal relationships emerges only when we come to birds and mammals

We have been told that viviparity evolved independently many times and in many different ways, in the vertebrate series It is nevertheless manifest that the central connecting link in the whole of this story is the corpus luteum I should like to know much more about the way the function of the corpus luteum becomes linked with that of the placenta but I doubt if I shall unless Prof Amoroso takes us more slowly along

the road 'from embryotroph to placenta One of the difficulties is that we lack a precise definition of the corpus luteum—or rather that it is impossible to decide in marginal cases whether or not a given structure is a corpus luteum in the sense that it has the function of a corpus luteum This particular difficulty does not only apply to the corpus luteum Dr Miller tells us that in certain species of lizard testicular interstitial cells may not necessarily be responsible for the secretion of androgen.

Variation in reproductive processes is revealed not only in their normal external manifestations but also in the influence which environmental factors have upon the sensitivity of tissues to different forms of hormonal stimulation This is the clear inference of Dr van Oordt s demonstration of the seasonal changes which occur in the sensitivity of the gonads to gonadotrophic stimulation And the conclusion is reinforced by what Dr Breneman and Dr Fraps have told us about the interaction of nutritional states and the responsiveness of tissues Such environmental factors should presumably be regarded as modulators of autonomous reactions and autonomous rhythms A question that arises is whether these rhythms are dominated by the pituitary gland or whether they are a function of all the tissues with which we have been concerned We are all taught that the alternation of FSH and LH activity of the anterior pituitary interacts with cyclical changes in the gonads What Dr Witschi has said at this meeting suggests however that the alternation in pituitary function may continue even in the absence of the gonads This observation interests me greatly since there are clear indications in other experimental work that cyclical changes occur in the uterus of rats as well as monkeys after ovariectomy and that they are manifestations of cyclical variations in the secretion of minimal amounts of sex hormones by the adrenal cortex

It is of course conceivable that all tissues undergo cyclical changes in activity and responsiveness even though these changes do not necessarily have the same period From what Dr Witschi has said it is also clear that while seasonal changes in secondary sexual characters are normally under the immediate control of the gonads this is not always so His observation that seasonal changes in the coloration and plumage of the weaver finch persist even after its gonads are removed lends itself to no other conclusion

Our discussion has not only dealt with external factors as modulators of autonomous rhythms and reactions it has also shown that the reproductive activity of the organism is immediately affected by the environmental changes associated with the seasons Prof Benoit Dr Marshall and Dr Breneman have indicated how important a factor light is in birds and mammals Others have shown that temperature has a major effect in the case of fish and amphibians In considering these facts we have as I suggested during the course of the discussion to be clear about two distinct propositions The first is that reproductive phenomena manifest periodicity regardless of environmental factors This generalization is illustrated by Prof Benoit s observation that blind drakes or drakes kept completely in the dark undergo seasonal testicular development even though this occurs more slowly than the corresponding process in drakes maintained in the light A corresponding observation has been made in my own laboratory Blind

ferrets that are kept from year to year will come into heat in the spring at the same time as do normal animals an observation which merely confirms a conclusion that was drawn many years ago by Parkes from experiments in which ferrets were kept in a dark cupboard

The second proposition is that autonomous rhythms may be affected by changes in the external environment Thus the spring oestrus can be accelerated by several months in anoestrous ferrets—or drakes—that are exposed in the late autumn and early winter to artificial light This in a sense is what one would expect Reproductive phenomena are processes that unfold in time they are in consequence cyclical It follows that the autonomous physiological rhythms they represent and which have become selected in the evolutionary sense must continue to be sensitive to environmental change If this were not the case the species manifesting them would have died out in the course of geological time—for the good reason that the seasons themselves are not immutable from the point of view of the calendar To take an extreme case an animal which breeds in March and April in the northern hemisphere would die out south of the equator if it were to continue breeding in the same months of the year

These facts pose fascinating physiological problems which have been touched on by Dr Fraps and Prof Benoit We want to know how the environmental changes that are registered on the surface receptors of the organism influence the behaviour of the pituitary gland and in turn that of the gonads We want to know whether the same kind of mechanism is involved whatever sensory modality is concerned whether it be light or temperature Much work is being done on this problem of which Prof Benoit s studies are notable examples But my own view is that it is premature to draw any far-reaching conclusions There are far too many inconsistencies in the story How is it for example as Dr Leathem has told us that androgen can maintain the testis in hypophysectomized mammals whereas if the story told to us by Dr Fraps is carried to its logical conclusion progesterone affects the process of ovulation *via* the hypothalamus?

The wealth of observation with which we have been regaled at this conference indicates at all points that problems are not as simple as they sometimes seem or are made out to be The substrate on which hormones act may differ from moment to moment according to the time of the year according to the place according to the temperature light nutritional state and so on and according to the activity of the endocrine organs other than the one that is being studied The indices which reveal to us the operation of physiological mechanisms or which specify the action of hormones need to be constant as was illustrated to us only too clearly by Dr Leathem s discussion of assays But the manifestation of these indices varies considerably in different vertebrates and the variation is not only between species but also as Prof Gallien Dr Jost and Dr Leathem have shown in different strains and at different ages in the same species Physical criteria might suggest that a steroid hormone and an apparently pure and crystalline pituitary hormone may be the same But when we try to define their physiological properties we are inevitably set back by differences in our test objects—in the same way as one would expect the harmony to vary as we move our hands in

the same way over different musical instruments This discussion of the comparative endocrinology of reproduction has shown us that we have a long way to go before we even know how our instruments the animals on which we work differ and that the task of finding out will prove highly rewarding

MEMOIRS OF THE SOCIETY FOR ENDOCRINOLOGY

No 1 The Thyroid Gland July 1953

No 2 The Determination of Adrenocortical Steroids and their Metabolites November 1953

No 3 The Technique and Significance of Oestrogen Determinations February 1955